This is a digital copy of a book that was preserved for generations on library shelves before it was carefully scanned by Google as part of a project to make the world's books discoverable online.

It has survived long enough for the copyright to expire and the book to enter the public domain. A public domain book is one that was never subject to copyright or whose legal copyright term has expired. Whether a book is in the public domain may vary country to country. Public domain books are our gateways to the past, representing a wealth of history, culture and knowledge that's often difficult to discover.

Marks, notations and other marginalia present in the original volume will appear in this file - a reminder of this book's long journey from the publisher to a library and finally to you.

Usage guidelines

Google is proud to partner with libraries to digitize public domain materials and make them widely accessible. Public domain books belong to the public and we are merely their custodians. Nevertheless, this work is expensive, so in order to keep providing this resource, we have taken steps to prevent abuse by commercial parties, including placing technical restrictions on automated querying.

We also ask that you:

+ *Make non-commercial use of the files* We designed Google Book Search for use by individuals, and we request that you use these files for personal, non-commercial purposes.

+ *Refrain from automated querying* Do not send automated queries of any sort to Google's system: If you are conducting research on machine translation, optical character recognition or other areas where access to a large amount of text is helpful, please contact us. We encourage the use of public domain materials for these purposes and may be able to help.

+ *Maintain attribution* The Google "watermark" you see on each file is essential for informing people about this project and helping them find additional materials through Google Book Search. Please do not remove it.

+ *Keep it legal* Whatever your use, remember that you are responsible for ensuring that what you are doing is legal. Do not assume that just because we believe a book is in the public domain for users in the United States, that the work is also in the public domain for users in other countries. Whether a book is still in copyright varies from country to country, and we can't offer guidance on whether any specific use of any specific book is allowed. Please do not assume that a book's appearance in Google Book Search means it can be used in any manner anywhere in the world. Copyright infringement liability can be quite severe.

About Google Book Search

Google's mission is to organize the world's information and to make it universally accessible and useful. Google Book Search helps readers discover the world's books while helping authors and publishers reach new audiences. You can search through the full text of this book on the web at `http://books.google.com/`

UC-NRLF
B 3 958 669

Digitized by Google

LIBRARY
OF THE
UNIVERSITY
OF
CALIFORNIA

Chemistry Lib.

BOUGHT FROM

SIGILLVM · VNIVERSITATIS · CALIFORNIENSIS
LVX
FIAT
MDCCCLXVIII
EX LIBRIS

CHEMISTRY LIBRARY

Digitized by Google

Digitized by Google

Digitized by Google

THE JOURNAL

6257

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

EDITED BY

H. D. DAKIN, New York, N. Y.
E. K. DUNHAM, New York, N. Y.
LAFAYETTE B. MENDEL, New Haven, Conn.
A. N. RICHARDS, Philadelphia, Pa.
DONALD D. VAN SLYKE, New York, N. Y.
CLARENCE J. WEST, New York, N. Y.

WITH THE COLLABORATION OF

J. J. ABEL, Baltimore, Md.
STANLEY R. BENEDICT, New York, N. Y.
R. H. CHITTENDEN, New Haven, Conn.
OTTO FOLIN, Boston, Mass.
WILLIAM J. GIES, New York, N. Y.
L. J. HENDERSON, Cambridge, Mass.
REID HUNT, Boston, Mass.
W. A. JACOBS, New York, N. Y.
WALTER JONES, Baltimore, Md.
ANDREW HUNTER, Toronto, Canada.
P. A. LEVENE, New York, N. Y.
JACQUES LOEB, New York, N. Y.
A. S. LOEVENHART, Madison, Wis.
GRAHAM LUSK, New York, N. Y.
E. V. McCOLLUM, Baltimore, Md.
A. B. MACALLUM, Toronto, Canada.
J. J. R. MACLEOD, Cleveland, Ohio.
JOHN A. MANDEL, New York, N. Y.
A. P. MATHEWS, Chicago, Ill.
F. G. NOVY, Ann Arbor, Mich.
THOMAS B. OSBORNE, New Haven, Conn.
T. BRAILSFORD ROBERTSON, Berkeley, Cal.
P. A. SHAFFER, St. Louis, Mo.
A. E. TAYLOR, Philadelphia, Pa.
F. P. UNDERHILL, New Haven, Conn.
V. C. VAUGHAN, Ann Arbor, Mich.
ALFRED J. WAKEMAN, New Haven, Conn.

VOLUME XXXIII
BALTIMORE
1918

Digitized by Google

PUBLISHED BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH FOR THE
JOURNAL OF BIOLOGICAL CHEMISTRY, INC.

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

Digitized by Google

QP501
J72
v. 33
CHEMISTRY
LIBRARY

CONTENTS OF VOLUME XXXIII.

LYLE, WILLIAM G., and CURTMAN, LOUIS J. A new method for the detection of occult blood in stools 1
OSBORNE, THOMAS B., and WAKEMAN, ALFRED J. The proteins of cow's milk 7
McCLENDON, J. F. A new hydrogen electrode for the electrometric titration of the alkaline reserve of blood plasma and other frothing fluids 19
HENDERSON, YANDELL. Applications of gas analysis. IV. The Haldane gas analyzer 31
HENDERSON, YANDELL, and SMITH, ARTHUR H. Applications of gas analysis. V. Blood gas analysis 39
HENDERSON, YANDELL. Applications of gas analysis. VI. The respiratory exchange and indirect calorimetry 47
McCOLLUM, E. V., and SIMMONDS, N. A study of the dietary essential, water-soluble B, in relation to its solubility and stability towards reagents 55
AUSTIN, J. HAROLD, and JONAS, LEON. Effects of diet on the plasma chlorides and chloride excretion in the dog 91
SAWYER, MARGARET, BAUMANN, L., and STEVENS, F. Studies of acid production. II. The mineral loss during acidosis: 103
LEVENE, P. A., and WEST, C. J. Lecithin. I. "Hydrolecithin" and its bearing on the constitution of cephalin 111
PALMER, WALTER W. The colorimetric determination of hemoglobin 119
VAN SLYKE, DONALD D. Gasometric determination of the oxygen and hemoglobin of blood 127
LUNDSGAARD, CHRISTEN. Studies of oxygen in the venous blood. I. Technique and results on normal individuals 133
VOEGTLIN, CARL, and SHERWIN, CARL P. Adenine and guanine in cow's milk 145
HOGAN, ALBERT G. The nutritive properties of kafirin 151
JEWELL, MINNA E., and LEWIS, HOWARD B. The occurrence of lichenase in the digestive tract of invertebrates 161
HUNTER, ANDREW, and CAMPBELL, WALTER R. The amount and the distribution of creatinine and creatine in normal human blood .. 169
KURIYAMA, SHIGENOBU. The influence of thyroid feeding upon carbohydrate metabolism. I. The storage and mobilization of the liver glycogen in thyroid-fed animals 193
KURIYAMA, SHIGENOBU. The influence of thyroid feeding upon carbohydrate metabolism. II. The epinephrine content of the adrenals of thyroid-fed rats 207

M589502

Digitized by Google

KURIYAMA, SHIGENOBU. The influence of thyroid feeding upon carbohydrate metabolism. III. The acidosis in experimental hyperthyroidism and its relation to epinephrine in the blood and the decrease of liver glycogen..... 215
LEVENE, P. A. The structure of yeast nucleic acid. II. Uridinephosphoric acid. Plate 1..... 229
McCRUDDEN, F. H., and SARGENT, C. S. The determination of sodium and potassium..... 235
OSBORNE, THOMAS B., and WAKEMAN, ALFRED J. Some new constituents of milk. Third paper. A new protein, soluble in alcohol.. 243
WATANABE, C. K. Studies in the metabolic changes induced by administration of guanidine bases. I. Influence of injected guanidine hydrochloride upon blood sugar content..... 253
BARNETT, GEORGE D. Mathematical formulas and acid excretion.... 267
VAN SLYKE, DONALD D. Studies of acidosis. X..... 271
FENN, W. O. The effects of electrolytes on gelatin and their biological significance. I. The effects of acids and salts on the precipitation of gelatin by alcohol..... 279
DANIELS, AMY L., and LOUGHLIN, ROSEMARY. Feeding experiments with peanuts..... 295
McCOLLUM, E. V., and SIMMONDS, N. A biological analysis of pellagra-producing diets. IV. The causes of failure of mixtures of seeds to promote growth in young animals..... 303
HART, E. B., and STEENBOCK, H. Thyroid hyperplasia and the relation of iodine to the hairless pig malady. I. Plates 2 and 3.... 313
OKADA, SEIZABURO. On the estimation of amino-acid nitrogen in the blood..... 325
HENDERSON, YANDELL, and HAGGARD, H. W. Respiratory regulation of the CO_2 capacity of the blood. I. High levels of CO_2 and alkali..... 333
HENDERSON, YANDELL, and HAGGARD, H. W. Respiratory regulation of the CO_2 capacity of the blood. II. Low levels of CO_2 and alkali induced by ether. Their prevention and reversal..... 345
HENDERSON, YANDELL, and HAGGARD, H. W. Respiratory regulation of the CO_2 capacity of the blood. III. The effects of excessive pulmonary ventilation..... 355
HENDERSON, YANDELL, and HAGGARD, H. W. Respiratory regulation of the CO_2 capacity of the blood. IV. The sequence of trauma, excessive breathing, reduced CO_2 capacity, and shock..... 365
MITCHELL, H. H., and ECKSTEIN, H. C. A foam inhibitor in the Van Slyke amino nitrogen method..... 373
DUBIN, HARRY. Studies of the blood fat and lipoids of the dog before and after the production of experimental anemia..... 377
ATKINSON, H. V. Animal calorimetry. The influence of mechanical work upon protein metabolism during the height of meat digestion in the dog. Fourteenth paper..... 379
HAMMETT, FREDERICK S. Notes on the direct determination of urea and ammonia in placenta tissue..... 381

Digitized by Google

McCrudden, F. H., and Sargent, C. S. Comparison of the glucose and cholesterol content of the blood 387
Hill, R. L. The analysis of milk secreted by a suckling doe kid 391
Magath, Thomas Byrd. The catalase content of *Ascaris suum*, with a suggestion as to its rôle in protecting parasites against the digestive enzymes of their hosts 395
Csonka, Frank A. The fatty acids in human blood in normal and pathological conditions 401
McCollum, E. V., Simmonds, N., and Parsons, H. T. A biological analysis of pellagra-producing diets. V. The nature of the dietary deficiencies of a diet derived from peas, wheat flour, and cottonseed oil 411
Levene, P. A. The structure of yeast nucleic acid. III. Ammonia hydrolysis 425
Jones, Horry M. A detailed method for the preparation of histidine 429
Osborne, Thomas B., and Mendel, Lafayette B. The growth of chickens in confinement. Plates 4 to 6 433
Fenn, W. O. The effects of electrolytes on gelatin and their biological significance. II. The effect of salts on the precipitation of acid and alkaline gelatin by alcohol. Antagonism 439
Falk, I. S., and Winslow, C.-E. A. The effect of potassium bromate upon enzyme action 453
Crozier, W. J. Cell penetration of acids. IV. Note on the penetration of phosphoric acid 463
Pitz, W. Studies of experimental scurvy. II. The influence of grains, other than oats, and specific carbohydrates on the development of scurvy 471
Clark, Ernest D., and Almy, Lloyd H. A chemical study of food fishes. The analysis of twenty common food fishes with especial reference to a seasonal variation in composition 483
Schmidt, Carl L. A., and Watson, Thomas. A method for the preparation of taurin in large quantities 499
Schmidt, Carl L. A., von Adelung, Edward, and Watson, Thomas. On the elimination of taurin administered to man 501
Levene, P. A., and Cretcher, L. H., Jr. Synthesis and oxidation of tertiary hydrocarbons 505
Folin, Otto, and McEllroy, W. S. Copper-phosphate mixtures as sugar reagents. A qualitative test and a quantitative titration method for sugar in urine 513
Folin, Otto and Denis, W. The determination of lactose in milk ... 521
Bradley, Harold C., and Nichols, M. Starr. Nitrogen content of bacterial cells. I. Method 525
Loeb, Jacques. Ionization of proteins and antagonistic salt action .. 531
Blish, M. J. A study of the non-protein nitrogen of wheat flour 551
Index to Volume XXXIII 561
Proceedings of the American Society of Biological Chemists i

Digitized by Google

Digitized by Google

A NEW METHOD FOR THE DETECTION OF OCCULT BLOOD IN STOOLS.

By WILLIAM G. LYLE and LOUIS J. CURTMAN.

(*From the Harriman Research Laboratory, The Roosevelt Hospital, New York.*)

(Received for publication, November 5, 1917.)

The finding of occult blood in stools is of great importance in the diagnosis of ulcer and cancer of the stomach. However, the usual chemical methods are so unreliable in their results that they are at present discredited by many surgeons and internists. A review of the literature of this subject shows not only that the number of reagents proposed is very large, but that there also exists a considerable number of procedures for the use of these reagents. The two most widely used are gum guaiac and benzidine. The phenolphthalein reagent is difficult to prepare and moreover is entirely too sensitive for ordinary use. In a previous paper[1] we have shown how the benzidine reagent may give erroneous results unless great care is exercised in cleaning all the apparatus as well as in the use of reagents of definite concentrations. In the routine examination of stools we have found it difficult to get good controls with benzidine; moreover, it is too sensitive. An alcoholic solution of gum guaiac possesses the following disadvantages: (1) Not being of fixed composition or purity, a solution of definite concentration cannot be readily prepared. (2) Gums from different sources vary greatly in their sensitiveness as well as in the stability of the blue compound formed.[2] (3) For the best results, solutions must be freshly prepared.

[1] Lyle, W. G., Curtman, L. J., and Marshall, J. T. W., *J. Biol. Chem.*, 1914, xix, 445.

[2] In this connection the following experiment will be of interest. Three specimens of gum guaiac purchased from different dealers were ground and separately dissolved in 95 per cent alcohol in the proportion of 1:60. 0.5 cc. of each of these solutions was added to 2 cc. of an acetic acid-ether extract of a stool and treated with perhydrol. The first did not give a positive test, the second gave only a faint test, while the third yielded a good blue

Digitized by Google

All the above objections can be overcome by the use of guaiaconic acid which is the active agent of the gum. The preliminary work of this investigation was carried out with Merck's guaiaconic acid; but as this reagent was difficult to purchase, and its cost high, we experimented with gum guaiac in the hope of preparing therefrom some substance which in keeping qualities and sensitiveness would be fully equal to the commercial preparation of guaiaconic acid and, moreover, would possess the additional merit of being readily and economically prepared. This we succeeded in doing.

Preparation of the Reagent.

The method of preparation based on a series of preliminary experiments[3] is as follows: 50 gm. of the ground crude gum guaiac were treated in a beaker with 20 gm. of KOH dissolved in 200 cc. of water. After thorough stirring, the mixture was filtered with the aid of suction through cotton spread out in a thin layer in a Buchner funnel. The residue was washed with water until the combined filtrate and washings approximated 1.5 liters. To the diluted KOH solution were added with constant stirring 21 cc. of glacial acetic acid which was run dropwise from a burette. The precipitate was allowed to settle, the supernatant liquid poured off, and the residue washed once with water by decantation. The precipitate was then transferred to a Buchner funnel and dried by suction as much as possible. The precipitate was gently heated (small portions at a time) in an evaporating dish when most of the water separated and was removed by filter paper. After the removal of the water, and while the mass was still plastic, it was drawn out into thin sheets. In this condition the material rapidly hardens and dries in the air. The dried masses were then ground, treated with 300 cc. of hot 95 per cent alcohol and the mixture was thoroughly stirred to prevent the formation of a gummy mass. In a few minutes a dark brown material separated in a flocculent condition. This was filtered

color but of short duration. Under the same conditions, our preparation gave a good blue color which lasted for some time. The experiment was repeated with the same results.

[3] We wish to acknowledge the assistance of Mr. A. G. Wikoff in the preparation of this substance.

Digitized by Google

off and the alcohol removed from the solution by distillation. The residue in the flask was treated with 20 gm. of KOH dissolved in water, diluted considerably, and precipitated as before with about 20 cc. of glacial acetic acid. The precipitate was filtered off and dried as described above, after which it was ground and kept in a desiccator. The weight of the material finally obtained was 30.3 gm. representing a yield of 60 per cent. The time required to make this preparation was 4 hours, the distillation of the alcohol being the most time-consuming of all the operations.

A solution containing 1 gm. of this preparation in 60 cc. of 95 per cent alcohol was prepared, kept in a glass-stoppered bottle of colorless glass, and its sensitiveness determined from time to time with a standard solution of blood. No appreciable difference in sensitiveness was observed at the end of several weeks. There is therefore no need for freshly preparing the solution in making the test.

Procedure for the Examination of Stools for Occult Blood.

After considerable experimentation, the following procedure was adopted. Approximately 10 gm. of the stool are transferred to a beaker, 25 cc. of distilled water are added, and the mixture is stirred until of uniform consistency. Over a low flame, the mixture is heated with constant stirring to boiling and kept at the boiling temperature for several minutes. After cooling, one-half of the mixture is transferred to a glass-stoppered bottle of 80 cc. capacity, 5 cc. of Merck's reagent glacial acetic acid and 25 cc. of ether are added, and the mixture is thoroughly shaken and allowed to stand for several minutes. In a test-tube, 2 cc. of the ether extract are treated with 0.5 cc. (1 : 60) of the preparation described in this paper and finally one to five drops of 30 per cent Merck's reagent perhydrol are added slowly from a pipette. A decided green, light or dark blue, or purple color indicates the presence of blood in quantity to be of clinical significance. Our experiments confirmed those of Buckmaster who first pointed out that boiling the blood was without influence on the test with guaiaconic acid.

Digitized by Google

Determination of the Sensitiveness of the Above Procedure.

To this end a series of standard blood solutions was prepared as follows:

Solution A.—1 cc. of freshly drawn human blood was diluted to 100 cc. in a volumetric flask with distilled water. A few drops of toluene were added as a preservative. 1 cc. of this solution contained 0.01 cc. of blood.

Solution B.—1 cc. of Solution A was made up accurately to 10 cc. giving a solution of strength 1 cc. = 0.001 cc. of blood.

Solution C.—1 cc. of Solution A was diluted to 20 cc., yielding a solution of strength 1 cc. = 0.0005 cc. of blood.

Solution D.—1 cc. of Solution A was diluted to 30 cc., giving a solution the strength of which was 1 cc. = 0. 0003 cc. of blood.

Stools Employed in the Tests.

The stools used in the following tests were obtained from patients kept on a meat-free and soup-free diet. They were all formed stools and when tested by the procedure already mentioned gave negative results. 10 gm. of a negative stool were weighed in a beaker, about 50 cc. of water added, and thoroughly mixed; the mixture was then made up with water to 100 cc. and again uniformly mixed. Separate 10 cc. portions of this mixture were respectively treated with definite volumes of the above standard blood solutions, thoroughly mixed, and heated to boiling for 10 minutes with occasional stirring on a hot plate. After cooling, the mixtures were stirred for 5 minutes with 3 cc. of glacial acetic acid and 12 cc. of ether. 2 cc. portions of the clear extracts were transferred to test-tubes, and treated with 0.5 cc. of guaiaconic acid solution and five drops of perhydrol. The following results were obtained.

The experiments in Table I were repeated three times, using different negative stools for each series, with the same results. It appears therefore that when 1 gm. is taken for the test our technique is capable of detecting 0.0001 cc. of blood or one part in 10,000.

It may be of interest to note that when 1 cc. of 3 per cent medicinal hydrogen peroxide was used in place of the five drops of 30 per cent perhydrol, very faint and in some cases negative

Digitized by Google

TABLE 1.

10 Cc. Portions Containing 1 Gm. of Stool.

No.	Blood solution.	Amount of blood in 1 gm. of stool.	Test.
		cc.	
1	0.5 cc. Solution A.....	0.005	Blue.
2	0.1 " " A.....	0.001	Greenish blue.
3	1.0 " " B.....	0.001	" "
4	0.5 " " B.....	0.0005	Light green.
5	0.1 " " B.....	0.0001	" "
6	0.1 " " C.....	0.00005	Negative.
7	0.1 " " D.....	0.00003	"
8	No blood.....	0.0	"

tests were obtained in extracts which gave unmistakably positive results with the usual quantity of perhydrol.

To determine whether any advantage was to be gained by boiling the stool with acetic acid before extraction, the following experiments were made. Four portions, of 9 to 10 gm. each, of a negative, formed stool were treated as follows:

(*a*) To one portion 25 cc. of distilled water were added. (*b*) To another portion 25 cc. of water containing 5 cc. of glacial acetic acid were added. (*c*) Same as (*a*) with the addition of 1 cc. of blood Solution A. (*d*) The same as (*b*) with the addition of 1 cc. of blood Solution A. All were boiled for 10 minutes and the regular procedure was applied with the following results:

a.....Negative.
b..... "
c.....Positive.
d..... "

The above experiments were repeated with the same results, showing that the preliminary boiling of the stool with acetic acid and water possesses no advantage over the adopted procedure of boiling first with a little water and subsequently extracting with acetic acid and ether.

An examination of the various foods and pills given to patients in the hospital was made to see if they gave the test for blood. The procedure was the same as that employed in the examination of stools. For analysis about 25 gm. of each food were taken and

Digitized by Google

sufficient of the pills to make one dose. Negative results were obtained with the following: American store cheese, custard, milk, prunes, oatmeal, farina, wheatena, bread (white), rice, egg, ice cream, condensed milk, compound C pills, A, B, and S, and A, B, and P, and laxative pills. A positive test was given by meat soup.

Application of the Method to Hospital Cases.

The method described in this paper was used in the examination of over 500 stools to be tested for occult blood with satisfactory results. Before carrying out the test, the patient was kept for at least 2 days on a meat-free and soup-free diet. Although a light green color indicated small quantities of blood, for clinical purposes, we chose to report such cases as doubtful and considered only those cases positive in which a dark green or blue color was obtained.

SUMMARY.

A new procedure for the detection of occult blood in stools is proposed, the chief features of which are:

1. The use of a new preparation derived from gum guaiac, which possesses the merit of being both stable and sensitive.
2. Specific directions for the preparation of the ether extract.
3. The use of perhydrol.

The method was applied to the examination of over 500 specimens with satisfactory results.

Digitized by Google

THE PROTEINS OF COW'S MILK.*

By THOMAS B. OSBORNE and ALFRED J. WAKEMAN.

With the Cooperation of Charles S. Leavenworth and Owen L. Nolan.

(From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.)

(Received for publication, November 22, 1917.)

Although casein has been the subject of numerous investigations during the past few years, very little attention has been given to the other proteins of milk, notwithstanding the fact that the published data regarding these are comparatively meager. The reason for this is doubtless based on the idea, which formerly seems to have been held generally, that since casein forms about 80 per cent of the total protein in milk, the other proteins have such a subordinate importance in nutrition that a special investigation of them is not important.

Since recent studies of the chemical constitution, as well as of the relative nutritive value of individual proteins, have shown such wide differences between several of them, and especially since the heat-coagulable protein of milk, the so called lactalbumin, has appeared to be preeminently adapted to the nutritional requirements of growing animals, it has seemed worth while to learn more than is now known respecting these proteins. This has furthermore become important in view of the now generally recognized fact that milk contains among its water-soluble constituents the so called vitamines, which are essential for the growth of young, as well as for the continued maintenance of adult animals.

In all attempts to discover the nature of the water-soluble vitamine in milk it is essential to know the properties and pro-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

Digitized by Google

portion of protein which remains in solution after removing casein and the heat-coagulable proteins, for until we know how to remove this we cannot expect to deal successfully with the non-protein nitrogenous constituents of milk. We have therefore devoted much time to a painstaking study of this problem.

No attempt will be made to review the literature relating to this subject which has appeared in widely scattered journals, for in most cases the published details are too scanty to permit a critical comparison of the results with one another, or with those which we have obtained. References to the greater part of this literature can be found in the summaries of the published data relating to the chemistry of milk.[1]

EXPERIMENTAL.

4 liters of skimmed milk, obtained in January from a mixed herd of a neighboring dairy, were saturated with ammonium sulfate and the precipitate was allowed to drain over night on a large folded paper. It was then ground in a Nixtamal mill with water, the volume brought to 3 liters, and stirred for some time until all was dissolved except a little fat. The solution was then filtered through a dense felt of paper pulp and the latter washed with water. The filtrate was opalescent, but transparent. The proteins were again separated by saturating with ammonium sulfate, redissolved in 4 liters of water, and the slightly turbid fluid was treated with about 600 cc. of 1 per cent sulfuric acid which caused the casein to separate sharply. After standing on filters over night in a cold room, protected by toluene, the casein contracted to a dense mass and retained relatively little of the solution.

The clear filtrate was gradually treated with ammonia until neutral to litmus, but as no precipitate formed, the absence of unprecipitated casein, of "acid albumin," and also of calcium phosphate was demonstrated. The neutral solution was acidified slightly with sulfuric acid and saturated with ammonium sulfate. The precipitate was pressed on filter paper, dissolved in water,

[1] Raudnitz, R. W., *Ergebn. Physiol.*, 1903, ii, 193. Rosenau, M. J., and others, *Bull. Hyg. Lab., U. S. P. H., No. 41*, 1908, *No. 56*, 1909. Lane-Claypon, J. E., Milk and Its Hygienic Relations, London, 1916.

Digitized by Google

and the solution filtered through a felt of paper pulp to remove traces of insoluble matter. The filtrate (about 1 liter) was again saturated with ammonium sulfate and the precipitate allowed to drain on filters over night. When dissolved in water, an opalescent, slightly acid, but transparent solution was obtained. A little of this when dropped into much distilled water gave a turbidity which disappeared on stirring, but no degree of dilution could be found at which a distinct separation of globulin took place. After saturating this solution with ammonium sulfate the precipitate was filtered out and pressed on filter paper over night under a heavy weight. By the foregoing treatment the precipitate, which consisted of all of the milk proteins except those removed by acidifying the milk, was quite thoroughly freed from lactose and other non-protein constituents of the milk. The crumbly precipitate was then dissolved in water, and, in order to remove globulin, its solution was saturated with magnesium sulfate. The resulting precipitate, A, was filtered out and treated as will be described on page 11.

Lactalbumin.

A sample of the filtrate from the globulin, which was perfectly neutral to litmus, was diluted with three volumes of water and heated to 90°. A flocculent coagulum separated very slowly. Another sample, when made distinctly acid to litmus with acetic acid, behaved in the same way. The remaining solution was diluted with three volumes of water, made slightly acid to litmus with acetic acid, and heated to boiling. The coagulum was filtered out, washed with boiling water till freed from sulfate, then with dilute, and finally with absolute alcohol, and dried over sulfuric acid. This preparation of coagulated lactalbumin, which had been freed from globulin by saturating its neutral solution with magnesium sulfate, weighed 10.8 gm., contained 6.25 per cent of moisture, and 0.16 per cent of ash. Its composition, ash- and moisture-free, after drying at 110°, was:

Digitized by Google

Lactalbumin Free from Globulin.

	Coagulated.			Sebelien.[2]
	I	II	Average.	
Carbon	52.61	52.42	52.51	52.91
Hydrogen	7.18	7.01	7.10	7.18
Nitrogen	15.38	15.48	15.43	15.77
Sulfur	1.92		1.92	1.73
Phosphorus	Trace.		Trace.	0.18
Oxygen (by difference)			23.04	
			100.00	

In harmony with the fact that this coagulated preparation of lactalbumin contained only a trace of phosphorus we found that another preparation which had been similarly precipitated by saturation with magnesium sulfate, but had not been coagulated by heat, when extracted with alcohol yielded only an insignificant amount of substance resembling phosphatide, and that this contained only a trace of phosphorus. It is thus evident that the phosphatide, which we previously obtained from the coagulated protein of milk,[3] is not associated with the purified lactalbumin.

The filtrate from our coagulated lactalbumin gave a slight flocculent precipitate with acetic acid and potassium ferrocyanide, showing the presence of a very little still uncoagulated protein. That a very complete separation of globulin from albumin is obtained by the method employed in making this preparation is shown by the results of anaphylaxis experiments made by H. G. Wells with another preparation made in the same way, except that the final precipitation of the albumin was effected by *acidifying* the filtrate from the globulin precipitated with magnesium sulfate, instead of by heating. This precipitate was freed from most of the adhering solution of magnesium sulfate by pressing between layers of filter paper, and was then dried over sulfuric acid. The preparation thus obtained, which still contained much magnesium sulfate, was perfectly soluble in water. Guinea pigs sensitized by this preparation were promptly killed by a

[2] Sebelien, J., *Z. physiol. Chem.*, 1885, ix, 463.

[3] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1915, xxi, 539.

Digitized by Google

second injection. On the other hand, those sensitized with a preparation of the carefully purified globulin showed no reaction to a subsequent injection of lactalbumin, nor were they protected against a later injection of the globulin, for animals thus treated died within a few minutes.

Assuming that 1 liter of whole milk, containing 3.5 per cent of fat, is equal to 965 cc. of skimmed milk, the 10.11 gm. of ash- and moisture-free coagulated lactalbumin obtained in this experiment from 4 liters of skimmed milk are equal to about 2.4 gm. per liter of the original whole milk. That the actual amount of lactalbumin was somewhat greater than this is indicated by facts discussed later in this paper in connection with the possible presence of proteoses in milk.

Lactoglobulin.

In order to remove completely lactalbumin from precipitate A (page 9), which was produced by saturating the neutral solution with magnesium sulfate, this was dissolved in about 400 cc. of water, the solution again saturated with magnesium sulfate, and the precipitate filtered out and pressed very thoroughly between filter paper. After repeating this process the final precipitate, when dissolved in about 250 cc. of water, yielded a brownish colored, strongly opalescent, but transparent solution which was just perceptibly acid to delicate litmus paper. Dropped into much distilled water a cloud formed suggesting precipitation, but on mixing only a turbidity remained. When 3 cc. of this solution were mixed with 3 cc. of water 4 or 5 drops of 1 per cent acetic acid and 0.5 cc. of 20 per cent sodium chloride solution, a coagulum appeared on heating to 71°, which separated in much finer flocks than those yielded by heating a solution of lactalbumin. After heating at 71–75° for a short time the coagulum was filtered out. The filtrate gave no turbidity on boiling but did give a slight precipitate when saturated with ammonium sulfate.

The main solution was accordingly diluted with water to 600 cc., made distinctly acid to litmus with 3 cc. of 1 per cent acetic acid, and 30 cc. of a 20 per cent sodium chloride solution were added, which caused opacity in thin layers, but produced

Digitized by Google

no visible particles. On heating to 72° a flocculent coagulum formed which separated so imperfectly that centrifugation was necessary for its removal. The deposit was washed free from sulfate by centrifuging several times with water, then with 50 per cent alcohol, dehydrated with absolute alcohol, extracted with ether, and dried over sulfuric acid. This preparation weighed 2.4 gm. and contained 8.55 per cent of moisture (dried at 110°) and 2.12 per cent of ash. This is equivalent to 0.52 gm. of moisture- and ash-free globulin per liter of whole milk, assuming that 1 liter of whole milk is equivalent to 965 cc. of skimmed milk. Ash- and moisture-free, this preparation had the following composition:

Lactoglobulin Free from Lactalbumin.

	I	II	Average.
Carbon	51.92	51.85	51.88
Hydrogen	7.01	6.88	6.96
Nitrogen	15.47	15.40	15.44
Sulfur	0.86		0.86
Phosphorus	0.24		0.24
Oxygen (by difference)	.....	.....	24.62
			100.00

Since all of the phosphorus contained in this preparation was recovered in the ash, special investigation was required to determine whether this belonged to phosphatides, to contaminating phosphates, or to other phosphorus-containing substances. Accordingly another preparation was made from 6 liters of centrifugated milk in the same way up to the point of final precipitation with magnesium sulfate. When freed from almost all of the adhering solution by pressing with filter paper, it was reduced to a fine powder and digested for a long time with absolute alcohol. Dried over sulfuric acid this preparation weighed 7.72 gm. and contained 7.21 per cent of nitrogen, equal to 3.6 gm. of globulin, the balance being magnesium sulfate and water. The alcoholic extract when evaporated left a residue weighing 0.1960 gm., thus making the total amount of the magnesium sulfate precipitate 7.92 gm. The residue from the alcoholic

Digitized by Google

extract was treated with ether, which dissolved all but a little finely divided white substance. This latter had the appearance characteristic of the diaminophosphatide which we previously obtained under similar conditions from the mixed phosphatides extracted from the coagulated milk protein.[3] Without filtering, the ether solution was poured into acetone and yielded a precipitate which contained phosphorus equal to about 0.0800 gm. of phosphatide, equivalent to 2.2 per cent of the lactoglobulin as calculated from the nitrogen in the magnesium sulfate precipitate after extraction with alcohol.

The preparation of lactoglobulin coagulated by heat and extracted with alcohol contained 0.24 per cent of phosphorus, which would correspond to about 7 per cent of phosphatide if all of the phosphorus were present in such substances. Owing to the presence of the relatively large amount of magnesium sulfate it was not possible to determine how much of this phosphorus belonged to inorganic phosphates, but the repeated precipitations with ammonium sulfate ought to have removed this completely.

As just noted, the alcoholic extract of the uncoagulated lactoglobulin contained phosphorus equal to 2.2 per cent of the globulin. In this respect a similarity exists with the vitellin from hen's eggs which on treatment with alcohol yields over 20 per cent of substance, much of which is phosphatide, while the thoroughly extracted protein contains about 1 per cent of phosphorus. It is possible that lactoglobulin is a similar lecithalbumin, or is a mixture of proteins, one or more of which belongs to this group. Further investigations are needed before final conclusions can be reached as to the true nature of this protein which, in its solubility relations, has little in common with most other globulins.

These results, together with the fact that the coagulated lactalbumin as well as the albumin fraction after repeated precipitation with magnesium sulfate in the uncoagulated state yielded only a trace of phosphorus, show that phosphatides are associated only with the lactoglobulin.

Preliminary tests showed that the filtrate from the coagulated globulin contained protein which, even after adding more sodium chloride or acetic acid, could not be caused to separate by longer heating. A liberal quantity of alcohol therefore was

Digitized by Google

added to the filtrate from the coagulated globulin, and the precipitate, which separated after long standing, was filtered out and digested with water. Since the resulting gelatinous mass could not be washed on filter paper it was suspended in about 400 cc. of 90 per cent alcohol, centrifuged, washed with absolute alcohol, and dried over sulfuric acid. This preparation (lactoglobulin?) weighed 0.97 gm., equal to about 0.2 gm. per liter of the original whole milk.

The alcohol which had been in contact with this smaller part of the magnesium sulfate precipitate was evaporated and the residual solution shaken out with ether. The concentrated ethereal extract was poured into acetone, and a small precipitate which had the appearance of a phosphatide was obtained.

Proteoses in Milk.

We have devoted a great deal of time to determine definitely whether or not proteoses are really present in milk, for this question is not only of interest to the physiologist, but has importance in devising methods for future investigations of the non-protein nitrogenous constituents of milk, as well as for making preparations of our so called protein-free milk which shall contain less protein than the product heretofore employed. Since our numerous experiments have yielded no results from which positive conclusions can be drawn only a general account of their outcome need be given here.

By proceeding according to the conventional methods, first removing casein by precipitation with acid, and albumin and globulin by boiling the acid filtrate from the casein, some protein has always been found in solution. Repeated attempts to obtain sufficient quantities of this protein for detailed examination showed that not only its properties, but also its proportion varied greatly in different experiments, and in many ways it differed distinctly from typical proteoses. Our experience has convinced us that the protein thus escaping coagulation is largely derived from some one or other of the milk proteins through the action of the reagents used. That it is not the result of the action of enzymes or of bacteria in the milk seems to be excluded by the fact that centrifugated milk, saturated with ammonium sulfate

Digitized by Google

within an hour after drawing from the cow, gave a precipitate which, when worked up as rapidly as possible, yielded substantially the same result as did milk 12 hours old when similarly treated. The acidity of the filtrate from casein, precipitated by either hydrochloric, or by acetic acid, in the presence, or absence, of phosphates, or of sulfates is pH = 4.6[4] so that coagulation in most of our experiments took place at practically the same degree of acidity. At this degree of acidity some alteration of the globulin or albumin may take place during the heating necessary to coagulate these proteins. We have found however, that if fresh milk is heated to boiling before acidifying and casein together with the coagulated proteins is subsequently removed by acidifying and filtering, a small amount of uncoagulated protein still remains in solution.

No method that we have as yet devised has enabled us to obtain evidence that a certain but relatively very small amount of proteose may not be an original constituent of cow's milk. In our last attempt we obtained per liter of milk only 0.2 gm. of protein resembling proteose although mechanical losses were at the most very small. Until the conditions under which coagulation of the globulin and albumin can be quantitatively effected are established we shall not be in a position to determine definitely whether or not milk contains any proteose.

Siegfried's "Nucleon."

Siegfried[5] obtained from the extract of horse muscle a substance which he called *Fleischsäure*. This he believed to occur in combination with phosphoric acid, which compound he first named *Phosphorfleischsäure*. Since *Fleischsäure* appeared to be identical with antipeptone,[6] Siegfried later proposed to call the phosphoric acid combination nucleon[7] on account of the similarity of its products of hydrolysis to those of the nucleins. Nucleon can be precipitated as an iron compound, carniferrin, which contains

[4] Cf. Allemann, O., *Biochem. Z.*, 1912, xlv, 346. Michaelis, L., and Pechstein, H., *ibid.*, 1912, xlvii, 260.

[5] Siegfried, M., *Ber. Sächs. Ges. Wissensch. zu Leipzig, Math. u. phys. Classe*, 1893, xlv, 485.

[6] Siegfried, *Arch. Physiol.*, 1894, 401.

[7] Siegfried, *Ber. chem. Ges.*, 1895, xxviii, 515.

Digitized by Google

about 30 per cent of iron, is soluble in an excess of alkali, and behaves like a compound of protein with iron.

Siegfried[8] later obtained a product from cow's milk which he considered to be identical with nucleon from muscle extract, except that it yielded fermentation lactic acid instead of paralactic acid, when hydrolyzed with baryta. He obtained this by precipitating casein with acid and the coagulable proteins by boiling. After removing phosphates by adding calcium chloride and ammonia, and neutralizing the filtered solution, carniferrin, the iron compound of nucleon, was precipitated by adding ferric chloride and boiling.

We have confirmed Siegfried's observation by applying the above procedure to milk serum from which the proteins and phosphates had been removed as he directs and much of the lactose by concentration and crystallization. This solution, however, contained a not inconsiderable quantity of proteose-like protein which could be separated by saturation with ammonium sulfate and readily obtained almost free from phosphorus by treating its solution with baryta, removing the latter with sulfuric acid, and precipitating with an excess of alcohol, a procedure substantially like that employed by Siegfried. Practically all of this proteose was included in the precipitate produced by adding ferric chloride and boiling. However, although a considerable amount of ferric chloride could be added to the filtrate from the precipitate produced by saturating with ammonium sulfate before a reaction with ferrocyanide occurred, no nucleon-like precipitate could be obtained in this solution freed from proteose. Although Siegfried makes no statement as to the presence or absence of proteose-like substances in solution after removing coagulable proteins it would appear that he did not realize that such were present. Since he took no step to remove these, our experience leaves little doubt that such protein formed a part of his carniferrin. It is to be noted that the aqueous extract of ox muscle when freed from coagulable proteins by boiling contains a small amount of protein which can be precipitated by saturating with ammonium sulfate.

Our work with milk has convinced us that a part of the phos-

[8] Siegfried, *Z. physiol. Chem.*, 1895–96, xxi, 360.

Digitized by Google

phorus of the serum is organically combined and that some of this, at least, is precipitated by ferric chloride under the above conditions, for a sample of the carniferrin precipitate which we obtained, when dissolved in cold dilute nitric acid, gradually deposited a voluminous light yellowish precipitate on standing at room temperature with no evidence of the formation of the characteristic yellow ammonium phosphomolybdate, whereas another sample did not give the yellow precipitate till boiled for some minutes and this gradually increased on continued boiling just as if phosphoric acid were being liberated by hydrolysis. That milk serum, freed from calcium phosphate by neutralizing, still contains phosphorus in organic combination is made probable by the fact that when this is acidified and boiled a precipitate of calcium phosphate forms when the solution is again made alkaline.

In view of these facts it is our opinion that the nucleon which Siegfried obtained from milk was probably a mixture of uncoagulable protein and some still unidentified organic substance which yields phosphoric acid on hydrolysis.

An Alcohol-Soluble Protein in Milk.

From the alcoholic washings of the large quantities of casein which have been made in this laboratory we have isolated several hundred grams of a protein which closely resembles gliadin of wheat in its solubility in alcohol of 50 to 80 per cent. Although the actual amount of this protein in milk is extremely small, the large quantity obtained as a by-product from the necessary processes incident to our feeding experiments seemed to justify a special investigation of its physical and chemical peculiarities. The results of this investigation, which will form the subject of a subsequent paper, have shown that this protein is an original constituent of milk and not a derivative of any of the other proteins contained therein. Since preparations have been obtained so far free from each of the other milk proteins that no evidence of such contamination could be detected by the anaphylaxis reaction, we feel justified in regarding it as a new constituent of this important food product.

Digitized by Google

Digitized by Google

A NEW HYDROGEN ELECTRODE FOR THE ELECTROMETRIC TITRATION OF THE ALKALINE RESERVE OF BLOOD PLASMA AND OTHER FROTHING FLUIDS.

By J. F. McCLENDON.

(From the Physiological Laboratory of the University of Minnesota Medical School, Minneapolis.)

(Received for publication, October 12, 1917.)

Van Slyke and Cullen have devised a practical method for the determination of the bicarbonate reserve of plasma, based on Van Slyke's method for determining total CO_2. The method presented in this paper also determines the bicarbonate, but by titration of the alkali instead of by the determination of the CO_2 of the $NaHCO_3$ molecule.

At present the most accurate method of titrating the blood alkali against acid to a definite end-point is afforded by the gas chain, since the end-points of indicators are rendered indefinite by the proteins present. With the electrometric technique one may choose between two modes of titration, (1) adding a definite amount of acid and measuring the change in pH, or (2) as in ordinary alkalimetry, adding as much acid from a burette as is necessary to obtain a definite pH. Cullen (1917) in a recent paper has used the former method. The latter, however, has the advantage that it permits one to choose an end-point so near the pH of circulating blood that comparatively little of the acid added combines with proteins and phosphates, so that only the bicarbonate is titrated.

In this paper apparatus is described for a convenient electrometric titration by addition of acid until a desired end-point is reached. As end-point we have chosen the pH of water, which is 7.00 at 23°. During the titration the free carbonic acid is reduced to approximately zero by washing out with hydrogen gas. Under these conditions (pH = 7.0, H_2CO_3 concentration = 0) all the

Digitized by Google

$NaHCO_3$ is changed to NaCl before the end-point is permanently attained; *i.e.*, for pH = 7 the ratio $\frac{H_2CO_3}{NaHCO_3}$ must be about $\frac{1}{7}$. If, by washing out the CO_2 gas, the H_2CO_3 is reduced to an infinitesimal value, the $NaHCO_3$ is also reduced to approximately zero.

The effect of the titration therefore is to add sufficient HCl to change all the $NaHCO_3$ into NaCl. The phosphates of the serum have a negligible effect on the titration. They are normally only about $\frac{1}{10}$ the molecular concentration of the bicarbonate, and change of pH from 7.4 to 7.0 changes only about one-fifth of the phosphate present from $NaHPO_4$ to NaH_2PO_4. The neutralizing power exerted by the serum proteins for the same pH change is calculated by Henderson to be equal to that of about 0.001 N alkali, while the bicarbonate is normally about 30 times as concentrated. Since the effect of the chief known buffers of the serum (aside from bicarbonate), *viz.*, phosphate and proteins, on the titration is calculated at only $\frac{1}{150}$ and $\frac{1}{30}$ respectively of the bicarbonate effect, the assumption appears justified that our titration measures the serum bicarbonate with a very slight error. As a matter of fact, we find that the amounts of acid required in our electrometric titration of normal human serum are equivalent to an alkali concentration of 0.030 N, which corresponds almost exactly to the average bicarbonate concentration of normal plasma (65 volume per cent CO_2 = 0.029 N) found by Van Slyke and Cullen as a result of direct gasometric determination of the CO_2 portion of the bicarbonate molecule.

In titrating plasma, I have used the rotating electrode (McClendon, 1917, *b*) thus avoiding frothing and eliminating the necessity of pumping, but it was necessary to disconnect the electrode every time more acid was added. It seemed necessary, therefore, to devise an electrode into which acid could be run and through which hydrogen (or H_2-CO_2 mixture) could be run without disconnection from the potentiometer.

After some preliminary forms of electrode had been tried, the rotating electrode shown in Fig. 1 was found satisfactory. The electrode vessel is cylindrical (30 × 36 mm.) with an opening 10 mm. in diameter in one end and the other end cemented to a cork pulley with sealing-wax, and the whole mounted in a wooden frame so as to rotate on a horizontal axis. Through the open end

Digitized by Google

of the cylinder is passed a large glass tube that fits snugly in the hole. Inside the large tube, three small glass tubes and a rubber tube are passed and cemented in with sealing-wax. The rubber

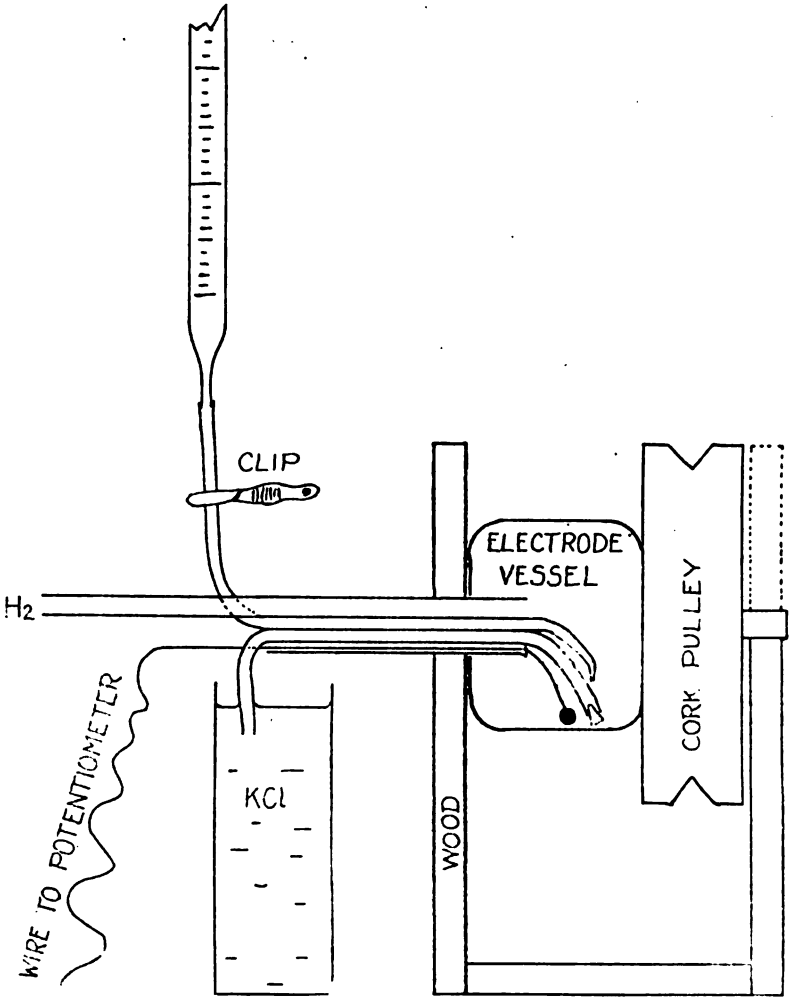


FIG. 1.

tube is 1 mm. bore and filled with a saturated KCl solution. The inner end hangs down in the cylinder and is closed with a bit of match that had been boiled in saturated KCl solution. The

Digitized by Google

outer end dips into a reservoir of saturated KCl solution connected with the saturated KCl-calomel electrode. The electrode proper is of gold coated with palladium black and connected to a platinum wire fused in one of the glass tubes. The wire is connected with the potentiometer. Lead glass was not used in sealing in the platinum wire owing to danger of reduction of lead thus making the platinum brittle. The 1 mm. glass tube was narrowed at one end so that the platinum wire would just go through. After insertion of the wire, a moment's heating in a tiny blast flame sealed it in the end of the glass tube. About 12 mm. of the platinum wire were allowed to protrude and a gold bead was fused on the end of it. This was accomplished by holding the end of a gold wire in a small Bunsen flame until a bead of the proper size formed, and advancing the end of the platinum wire into the flame until it touched the gold bead, then withdrawing it quickly. The gold bead was hammered to a disc. The finished electrode was put in place so that the gold disc hung down in the electrode vessel. Another glass tube, 1 mm. bore, was drawn to a dropping tip inside the electrode vessel and the outer end connected with a rubber tube of 1 mm. bore closed by a Langenbeck clip and connected with a 1 cc. pipette graduated in hundredths and filled with 0.1 N HCl or NaOH and used as a micro-burette. A glass tube of about 3 mm. bore served for the admission of hydrogen.

In setting up the apparatus, the frame was set in place and weighted with lead so as to remain in position. 1 cc. of plasma was run into the electrode vessel and it was put in place by inserting the axle of the cork pulley in the vertical slot in the frame made to receive it. The cork pulley was connected by means of a string belt to a Tiffany motor. The micro-burette and connecting tube were filled with HCl and the rubber tube was filled with a saturated solution of KCl. The gold disc was coated with palladium black by electrolysis with a 2 volt current and a fairly strong solution of palladium chloride. When this solution became yellow it was evaporated until it appeared light brown in a layer about 2 cm. thick. The electrode was sprayed a moment with distilled water and the 10 mm. glass tube containing the four smaller tubes carefully inserted through the hole in the wooden frame and the hole in the electrode vessel. The 10 mm. tube fits tight in the hole in the frame and, owing to the downward

Digitized by Google

bending of the small tubes, cannot be shoved straight in but must be started at an angle, and during the process the disc must not be allowed to dry or to touch any solid object. The operation is easier than it may seem. The 3 mm. glass tube is connected with a supply of hydrogen washed with $HgCl_2$, alkaline pyrogallol, and distilled water, and a vigorous stream of the gas passed through the electrode vessel. The Tiffany motor is started and the electrode vessel rotated so as to spread the plasma on its walls, at the same time leaving enough in the bottom to cover the disc. If any portion of the disc dries or gets a clot on it, it must be cleaned and recovered with palladium black. Readings are taken with the potentiometer and acid is run in while the vessel rotates. A 455 ohm galvanometer was used and a balance with the potentiometer took but a few seconds. The palladium black was removed each day by inserting the disc for a moment in aqua regia and spraying with distilled water, and was then deposited again. Since this can be done so quickly and without disconnecting the pipette or hydrogen tube or emptying the rubber tube of a saturated solution of KCl, I have not attempted to determine the length of life of a coating of palladium black. Platinum black has lasted longer in other electrodes but is not so easily removed. The gold disc may be dispensed with, but in order to get surface it is well to use a thick platinum wire, and a thick wire is liable to cause the 1 mm. glass tube to crack. I tried sealing a thick platinum wire into the glass tube with paraffin, and found that it worked for a few determinations. It could be coated with platinum black and cleaned by pulling it out of the paraffin and holding it in a flame. In putting it back, if great care was not used to see that the paraffin seal was perfect, moisture sometimes got into the glass tube and threw out the readings. Some platinic chloride solution that had been used for conductivity electrodes and had perhaps been contaminated with heavy metals, when used to platinize the hydrogen electrode; gave erroneous readings.

The end-point chosen for the titration was the pH of pure water which is about 7.03 at 20°, 7.00 at 22–23°, and 6.95 at 25°. The titrations were made at 23°.

The time required for a titration is not prohibitive for routine work. The collection, oxalation, and centrifugation of the blood under precautions to avoid the loss of CO_2 must be done in any

Digitized by Google

method. Measuring 1 cc. of the oxalated plasma into the electrode vessel and cleaning and recoating the disc takes but a few moments. If 0.4 cc. of 0.1 N HCl is added at the start the titration back with alkali takes 4 minutes. If the titration is made with HCl the speed with which the pH rises after the addition of acid indicates the amount of acid that it is safe to drop in the next time. The following example shows the time required for titration to the nearest hundredth of a cc. The final two pH readings give a possible method for estimating to thousandths of a cc., but whether such an estimation can be duplicated has not been determined. In some cases a weaker HCl solution was used to ensure more accurate volumetric measurement. In the following table, since seconds are not recorded, in some cases, two operations are placed opposite the same minute.

Hour, p.m.	Burette reading.	pH	Hour, p.m.	Burette reading.	pH
3.49	0.1		3.54		6.96
3.50		7.50	3.55		7.00
3.50	0.2		3.56		7.02
3.51		7.30	3.56	0.31	
3.51	0.3		3.57		6.85
3.52		6.50	4.00		6.87
3.53		6.90	4.05		6.87

The titration in this case took 16 minutes and the alkaline reserve was found to be between 0.03 and 0.031 N. It is not always possible to find the end-point with only four additions of acid, but it is only after the last two burette readings that it is necessary to wait for the definitive pH to be reached. In the following titration six additions of acid were required but the time spent was the same.

Hour, a.m.	Burette reading.	pH	Hour, a.m.	Burette reading.	pH
10.15	0.1		10.21		7.05
10.16		7.4	10.21	0.32	
10.16	0.2		10.22		6.95
10.17		7.20	10.25		7.05
10.17	0.25		10.25	0.33	
10.18		7.10	10.26		6.85
10.18	0.30		10.30		6.85
10.19		6.70			

Digitized by Google

The whole of 15 minutes need not be spent in titration as it is possible so to regulate the readings that practically all of the last 10 minutes may be spent in doing other things.

In the above titrations, the pH of pure water was taken arbitrarily as the end-point. This method gives at least comparative values and probably is not far from the true end-point, since it should at least determine the bicarbonate. It is impossible to titrate the phosphates, as a pH of about 3 to 4 would be necessary to decompose them, and at this pH the proteins would bind acid. The isoelectric point of serum albumin is at pH = 4.7, and of serum globulin pH = 5.4, and these proteins should not bind acid or alkali at their isoelectric points. These proteins may bind some alkali at pH = 7 and therefore it might seem best to use the mean of their isoelectric points as the end-point for titration. It is necessary, however, to use about a third more acid in order to do this and it seems improbable that the proteins bind a fourth of the alkali at pH = 7. There may be other ampholytes in the plasma with very different isoelectric points, since the concentration of diffusible phosphates is not sufficient to account for this large acid-binding power. According to a rough calculation, the pH of distilled water under an atmosphere containing 5 per cent CO_2 should be near the isoelectric points of serum proteins. It would seem of interest, therefore, to compare the titration of plasma under hydrogen with that under hydrogen containing 5 per cent CO_2. Owing to the fact that no tanks were at hand and my gas mixer holds only 1 liter, I was not sure that equilibrium was reached. A sample of plasma was titrated while H_2 was passing and pH = 7.00 was maintained after 0.33 cc. of acid had been run in. By means of a 3-way cock, H_2 + 5 per cent CO_2 was substituted and the change in pH noted while a liter of the mixture passed through the electrode vessel. The pH gradually fell, finally reaching 6.3. Since this is still far removed from the isoelectric point of the proteins, it seems probable that some other buffer action is present but whether the phosphates could account for this buffer action was not determined.

In the electrometric titration of many solutions the end-point is marked by a more or less distinct angle in the pH curve and it was thought advisable to plot these curves for plasma. The curves plotted by Cullen are almost straight lines and therefore

Digitized by Google

it would be necessary to remove more of the CO_2 than is accomplished by Cullen's method in order to show an angle or an asymptote. This removal of CO_2 is rapid at first but becomes slower and slower.

In the following table the number of minutes between dropping in the acid and measuring the pH is recorded.

Min.	pH	Burette reading.	Min.	pH	Burette reading.
	8.30		00		0.3
00		0.2	1	7.20	
1	6.70		10	7.35	
5	7.00		15	7.35	
10	7.13		00		0.4
15	7.36		1	6.00	
20	7.50		5	6.05	
25	7.65		10	6.07	
35	7.93		15	6.07	
40	8.02				

The above table merely shows that 40 minutes is not enough for the elimination of the CO_2 when an appreciable amount of the alkali is left in the plasma. The following table shows a similar experiment.

Min.	pH	Burette reading.	Min.	pH	Burette reading.
	8.04		10	7.30	
00		0.10	15	7.31	
1	7.30		00		0.31
2	7.65		1	6.65	
4	7.80		5	6.73	
5	7.85		10	6.73	
10	7.90		00		0.35
15	7.92		1	6.15	
20	7.93		10	6.15	
30	7.94		00		0.40
00		0.20	1	5.45	
1	7.00		5	5.50	
2	7.45		00		0.50
10	7.55		1	4.75	
20	7.56		13	4.75	
00		0.25	00		0.60
1	7.10		1	4.40	
3	7.20		7	4.40	

Digitized by Google

In the above table the rate of rise of pH with washing out of CO_2 was slower than in the previous experiment, probably due to less rapid flow of hydrogen. Somewhere between pH 5.4 and 4.4 the proteins coagulated, presumably at the isoelectric point. This experiment shows the acid-binding power of the proteins after their isoelectric points have been reached by the slow change in pH on dropping in more acid, the last 0.1 cc. of acid changing the pH only 0.35.

It seems to be impracticable to determine the alkaline side of the titration curve of plasma on titration with acid, and a far better way is the addition of an excess of acid at first and titration with CO_2-free NaOH. In the following experiment, 0.4 cc. of 0.1 N HCl were added to 1 cc. of plasma and titrated with 0.1 N NaOH. The blood had been exposed to air and hence the alkaline reserve of the plasma was not normal.

NaOH	pH	NaOH	pH
cc.		cc.	
0.00	5.40	0.25	9.02
0.05	5.92	0.30	9.43
0.10	6.57	0.35	9.76
0.125	7.00	0.40	10.02
0.15	7.42	0.45	10.24
0.20	8.32	0.50	10.42

On plotting these data a logarithmic titration curve is produced which shows, however, considerable buffer effect. After the alkali is dropped in and well mixed by tilting the apparatus and by the rotation, a fall of potential of less than 2 millivolts is noted. This effect increases as the alkalinity increases and may be due to a slow process of combination of alkali with protein. No argument can be deduced from this curve to show that some other pH than 7 is a more logical end-point for titration. It may be noted that when the alkali equivalent of the acid is dropped in the pH rises to 10. Plasma should reach this pH on driving out the CO_2 with hydrogen, but it would take a long time to accomplish it.

On adding an excess of acid to drive out the CO_2 it is necessary to allow the vessel to rotate and a rapid stream of hydrogen to

Digitized by Google

flow about 5 minutes or more before beginning to titrate back with NaOH. The following table gives the minutes required, as shown by the rise in pH to an equilibrium.

Min.	pH	Min.	pH
1	4.75	5	5.40
2	5.15	6	5.40
3	5.30	7	5.40
4	5.35	8	5.40

Determinations may be made on 0.1 to 0.5 cc. of plasma using 0.01 N HCl or NaOH. If the plasma does not cover the disc, it must be diluted with 1 per cent KCl solution until it covers the disc.

No undoubted cases of acidosis have yet been studied although I have agreed to do so. Plasma of lowered alkaline reserve was obtained by allowing the CO_2 to escape from the blood before centrifugation. Two such experiments gave the alkaline reserve to equal 0.017 and 0.02 N respectively, but the alkaline reserve before exposure to air was not determined. As may be seen in the above pages, the normal seems to be about 0.03 if pH = 7 is taken as the end-point. I do not, however, know the average normal and merely suppose certain samples to be normal since they were taken from patients without symptoms of acidosis. Van Slyke and Cullen give the average normal chemically bound CO_2 as 65 volume per cent, which I interpret as 0.029 N (since 22.4 liters of CO_2 dissolved in 1 liter of 1 N NaOH would make a 1 N solution of $NaHCO_3$).

It was found that plasma kept for hours at room temperature and then placed in the refrigerator over night presented no difficulties the next day. Plasma kept 36 hours at room temperature poisoned the hydrogen electrode so that no readings could be made. No H_2S was detected in this plasma, and the substance affecting the electrode was not determined.

My thanks are due to members of the staff of the University Hospital for drawing samples of blood.

Digitized by Google

BIBLIOGRAPHY.

Buckmaster, G. A., *J. Physiol.*, 1917, li, 164.
Clark, W. M., *J. Biol. Chem.*, 1915, xxiii, 475.
Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 369.
Fox, C. J. J., *Tr. Faraday Soc.*, 1909, v, 68.
McClendon, J. F., Physical Chemistry of Vital Phenomena, 1917 (*a*), Princeton.
McClendon, *J. Biol. Chem.*, 1917 (*b*), xxx, 265.
McClendon, J. F., Shedlov, A., and Thomson, W., *J. Biol. Chem.*, 1917, xxxi, 519.
Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.
Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
Henderson, L. J., *J. Biol. Chem.*, 1909–10, vii, 29.

Digitized by Google

Digitized by Google

APPLICATIONS OF GAS ANALYSIS.

IV. THE HALDANE GAS ANALYZER.

By YANDELL HENDERSON.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, October 30, 1917.)

This apparatus has such marked advantages for precise analysis of gas mixtures containing less than 30 per cent of oxygen, CO_2, and other absorbable or oxidizable gases that it has become the standard instrument. Nevertheless it has several features in which experience in this laboratory has shown that it may be simplified with advantage. The form of apparatus shown in Fig. 1 is the result of this experience. The advantage of this form is not in its use during analysis,—for this involves no significant change,—but chiefly in the ease with which it can be taken apart and cleaned (a feature in which the usual form of the apparatus is a source of much annoyance and waste of time), and in the lessened liability to "frozen" cocks and breakage. It has a minimum of rubber connections and only one glass stop-cock. (I have also tried out an apparatus with connections of glass tubing of only 0.7 mm. bore, but have found this too small. The usual bore of 1.1 to 1.3 mm. is best.)

The particular features of this form of the apparatus are: (1) a 4-way glass stop-cock shown in Fig. 2; (2) the adjustment of the volume of the control tube by means of a screw pinch-cock on a bit of rubber tubing on the lower end of the tube; (3) two large test-tubes in which the absorbents KOH or NaOH and potassium pyrogallate[1] are placed. The fine adjustment of pressure-volume is made by raising or lowering the test-tube containing the alkali. For the oxygen absorption the test-tube containing the pyrogallate may be raised and lowered, to avoid

[1] Sodium pyrogallate (as described by Shipley, J. W., *J. Am. Chem. Soc.*, 1916, xxxviii, 1687) has not proved satisfactory in this instrument.

Digitized by Google

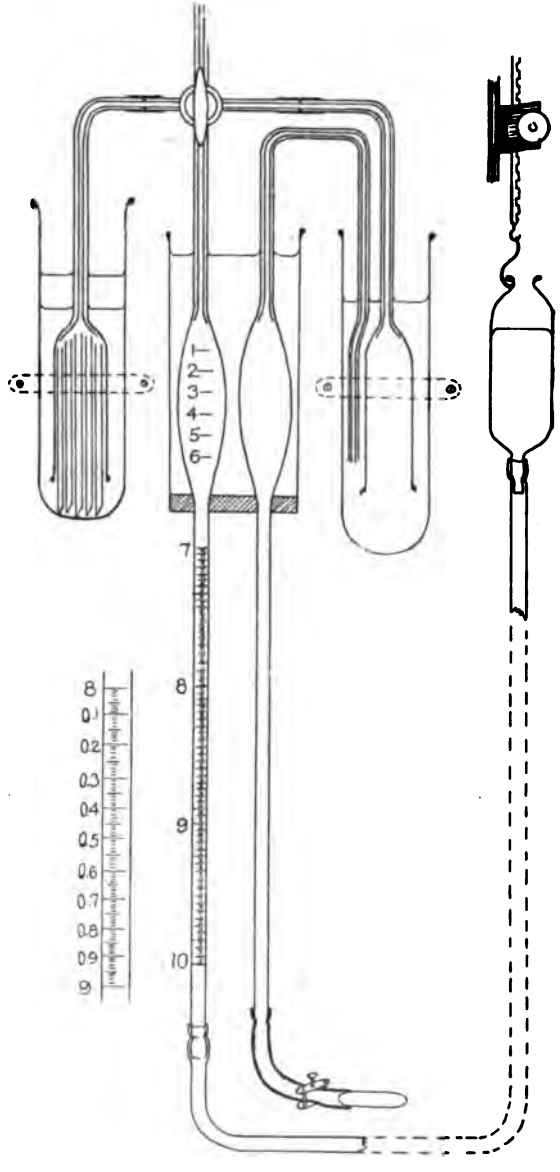


FIG. 1.

Digitized by Google

the fatiguing elevations of the mercury bulb. In use the pyrogallate solution is covered with a layer of petroleum oil sufficiently deep to protect it from the outside air. The lower ends of the pieces of glass tubing in the pyrogallate must be cut or broken diagonally. The control tube may be directly back of the gas burette instead of to one side as in the figure.

It will be noticed that the water jacket enclosing the gas burette and the control tube has been shortened so as to include little more than the

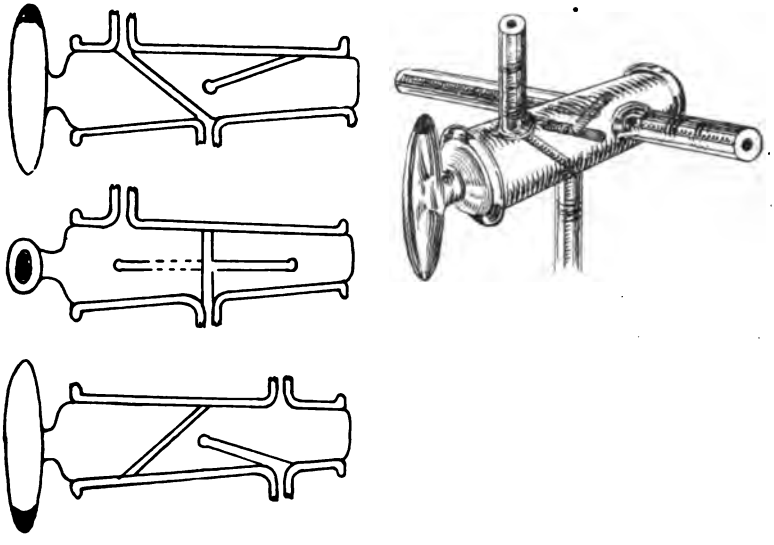


FIG. 2.

bulbs and upper parts. This is allowable because the object of the water jacket is not to keep the temperature constant but to make it possible by stirring or bubbling the water to bring the air in the two bulbs to the same temperature and water vapor tension at the time of an observation. It is extremely hard on the eyes to read a fine burette accurately through the glass and water of a long jacket. A small magnifying glass arranged to slide on the tube of the gas burette is convenient.[2] In all other respects the apparatus, the technique, and precautions on its use are the

[2] Wm. Gaertner and Company, 5345 Lake Park Avenue, Chicago, make a convenient form (Catalog No. C1365).

Digitized by Google

same as with the usual form of instrument.[3] Attention may however be called to one or two points which are often overlooked.

Red rubber should not be used on the connections where alkali may touch it, as it gives off sulfur which may finally appear as hydrogen sulfide in the burette.

To make the rubber connections tight (in case of leakage) it is convenient to wrap them with elastic bands. Loop the band around the rubber tube, pull tight, and wrap the free end around the tube several times. Pass a small curved forceps under the wrapping, catch the end of the rubber band over the points of the forceps, and twist the end under the wrapping by withdrawing the forceps. This is a much better method of making joints tight than wrapping them with wire.

The walls of the burette should always be moistened with a fraction of a drop of 1 per cent sulfuric acid, and 2 or 3 cc. of this fluid should be placed in the control tube.

The analyst must remember that accuracy in the use of the instrument does not depend upon an extreme effort to read the burette as finely as possible, but rather upon making sure that the absorptions are complete. It is essential therefore frequently after an analysis is supposedly complete to pass the gas over again into the absorbent and make another reading to be sure that no change occurs.

A check on calibration, tightness of joints, and efficiency of absorbents which should be used daily is to make an analysis of atmospheric air. If the apparatus is properly graduated and in good order the sum of the oxygen and CO_2 in uncontaminated atmospheric air should be found 20.96 per cent, with an allowable error of $\pm$ 0.03 per cent.

The method of graduation used by most American glass blowers is far too rough and inaccurate for the precision requisite for this instrument. Thus in some burettes of this type made in America I have found the errors of graduation ten or even twenty times as great as are allowable (giving an analysis of ordinary air at 21.20 per cent oxygen or worse). The absolute error of the instrument was only 0.1 cc. and this is not greater than is quite common and is allowable for common purposes in an ordinary titration burette. For the calibration of the gas burette the glass blower must not use water but mercury. The standard burette against which he calibrates must be of at least as fine bore as that of the tube of the burette to be calibrated. The standard burette must have been calibrated with weighed amounts of mercury and no mark on the burette purchased should have an error greater than 0.005 cc.

The purchaser of a burette should test it before assembling by inverting the burette, connecting it with a reservoir of mercury, filling to the 7, 8, 9, and 10 cc. marks, respectively, pouring this mercury into a small tarred beaker, and weighing. 1 cc. of mercury at room temperature (18°C.) weighs 13.55 gm. An allowance of 0.004 cc. may be made for the reversal of the convexity of the meniscus in the inverted burette.

---

[3] Haldane, J. S., Methods of Air Analysis, London, 1912.

Digitized by Google

The correctness of the graduation of the tube of the burette is most easily checked by drawing in on top of the mercury about 0.5 cc. of water. By raising and lowering the mercury and reading the air-water and water-

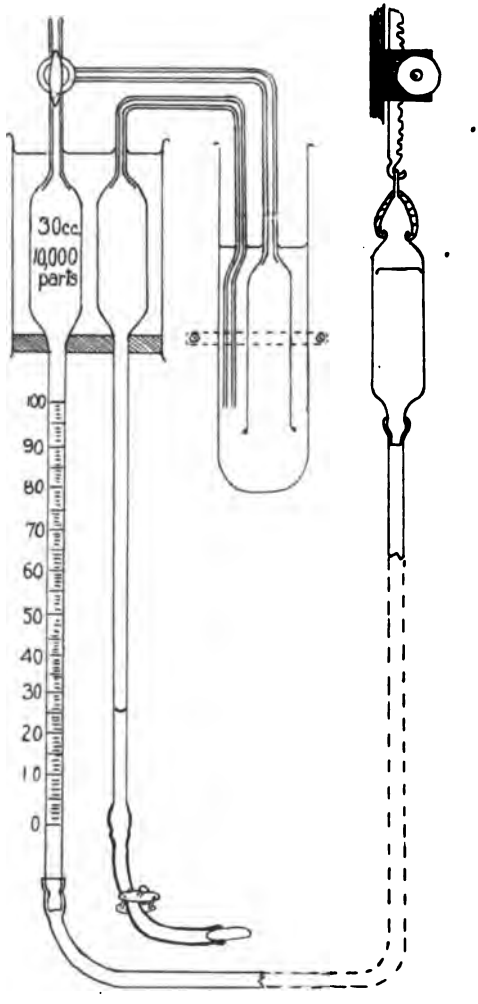


FIG. 3.

mercury menisci at various levels (allowing time for the water to drain from the sides), errors of graduation due to irregularities in the caliber of the tube are revealed. The difference in the volume between the two menisci should not vary more than 0.005 cc. at any two levels.

Digitized by Google

In Fig. 3 is shown a similar modification of the Haldane analyzer for the CO_2 content of atmospheric air. The bulb of the burette for this apparatus should have a capacity of 30 cc., and the tube of the burette should be graduated up to 100 parts (0.003 cc. each) of absorbable gas (CO_2) in 10,000 of air. This instrument is quite as accurate and much simpler and cheaper than the Pettersson-Palmquist analyzer.

In Fig. 4 is shown an apparatus which has been found useful for teaching purposes in this laboratory (one instrument to each six or eight students). For beginners it is a very much easier apparatus to use than the form shown in Fig. 1. Acidulated water (1 per cent sulfuric acid) is used in the burette instead of mercury. Common glass 6 ounce bottles are used to hold the absorbents, sodium hydroxide, and potassium pyrogallate. A layer of paraffin oil is placed on top of the absorbents to protect them from the air. The bottles stand on a shelf which also supports the water jacket of the burette.

The apparatus is used in the same manner as that described in the first paper of this series except that the addition of a control tube renders it possible to compensate for temperature changes in the course of an analysis. The use of the control tube is as follows. A strip of mm. paper is pasted on the leveling bottle. The top of the control tube is opened. The leveling bottle is lifted a few inches and the fluid allowed to rise part way up the control tube, the top of which is then closed. The leveling bottle is held so that the fluid is at the same level in the bottle and in the tube and a loop of wire (X) is placed at this height on the tube. Before each measurement of gas volume in the burette the leveling bottle is moved up or down the control tube, and by means of the mm. paper strip note is made of the height above or below the meniscus in the control tube at which the bottle must be held to bring the gas in the control tube to its original volume (at X). The leveling bottle is then held at the same height above or below the meniscus in the burette. In this way the gas in the burette can always be brought to the correct volume per molecule.

This instrument is adequate to all scientific and clinical investigations except those involving the most extreme accuracy. The elimination of mercury renders it very much easier to use.

Digitized by Google

If time is allowed for the fluid to drain from the walls of the burette before each reading oxygen analyses come out nearly as well as with the mercurial burette. With CO_2 the error due to

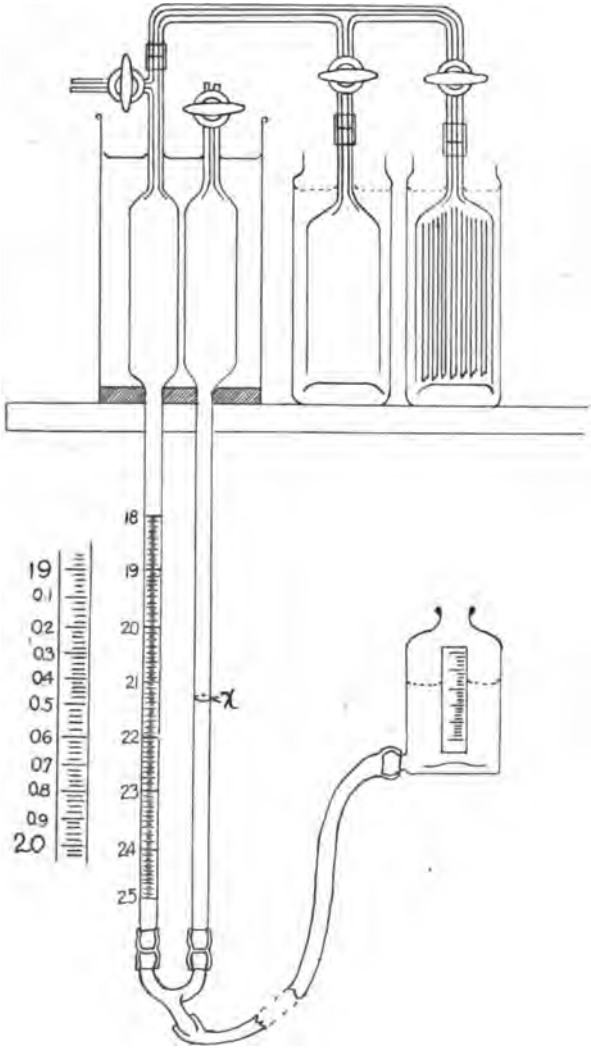


FIG. 4.

Digitized by Google

solution in the acidulated water is very close to 1 per cent; that is, analyses of air giving 5.50 per cent should be corrected to 5.55 per cent, or 2.10 to 2.12.

The following names may be used for the apparatus[4] described in this series of papers: (1) For that described in the first paper, analyzer for CO_2 in alveolar air and blood; (2) for that shown in Fig. 1 of this paper, respiratory gas analyzer; (3) for that in Fig. 3, atmospheric CO_2 analyzer; (4) for that shown in Fig. 4, modified Orsat analyzer.

I am indebted to Professor A. L. Prince and Mr. A. H. Smith for valuable assistance in the development of this apparatus.

[4] These instruments may be obtained from the Emil Greiner Company, 55 Fulton St., New York.

Digitized by Google

APPLICATIONS OF GAS ANALYSIS.

V. BLOOD GAS ANALYSIS.

By YANDELL HENDERSON and ARTHUR H. SMITH.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, October 30, 1917.)

The method to be described here is essentially a modification of that of Barcroft and Haldane (1) and of that described in the first paper of this series (2). The solutions employed are: (1) dilute ammonia (1 cc. of concentrated ammonia in 500 cc. of distilled water) kept tightly stoppered to avoid absorption of CO_2 from the air (or freed from carbonate with barium hydroxide and ammonium sulfate (3)). A little saponin is dissolved in the ammonia solution when an oxalate is used to prevent the blood sample from clotting. (2) A 10 per cent solution of potassium ferricyanide. (3) A 20 per cent solution of tartaric acid.

The apparatus used consists (1) of a Haldane gas analyzer in either its original or modified form (4), and (2) a small tube such as is shown in Fig. 1.

Oxygen Analysis.

The stop-cock of the diffusion tube is closed (*i.e.*, turned to connect the side and end nipples); 1.5 cc. of the ammonia solution are placed in the tube; and 1 cc. of the blood to be analyzed is introduced below the ammonia by means of a pipette. A short close fitting rubber stopper is inserted in the large end. A hypodermic needle (disconnected from its syringe) is passed through this stopper to allow the escape of the air compressed by the insertion of the stopper. The needle is withdrawn, and the tube is rotated gently to mix the blood and ammonia until the corpuscles are completely laked. A small all glass syringe fitted with a hypodermic needle is then filled with the ferricyanide solution and the air is expelled. The needle is then thrust through the

Digitized by Google

rubber stopper until the point projects in the interior of the tube, and 0.25 cc. of the ferricyanide solution are injected. The needle is then carefully withdrawn.

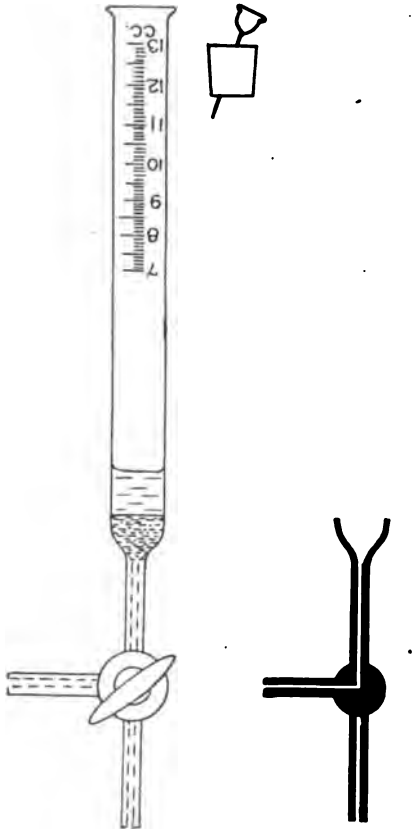


FIG. 1. The blood gas diffusion tube. The plug of the stop-cock is bored only in a right angle, not in a T, so that only two of the openings are connected at once. The open end of the tube is graduated as shown. This end of the tube is closed with a rubber stopper cut to about a half or a third the usual length, so that the needle of a small all glass hypodermic syringe can be thrust through it.

The tube is now placed for 5 minutes in a rotating apparatus (Fig. 2) consisting of a block of wood turning on an iron rod and belted to a slowly moving motor or shafting. There are a num-

Digitized by Google

ber of holes bored in the sides of the block of wood into which diffusion tubes can be inserted. The rate of revolution should be not more than 40 or 50 times a minute. During the rotation the contents of the tube spread in a thin film along the walls and allow complete diffusion of the liberated oxygen into the air of the tube.

The tube is next connected glass to glass with the gas analyzer by a short piece of heavy rubber tubing in the manner shown in Fig. 3. The mercury bulb of the analyzer is lifted until mercury runs out through the top of the analyzer and fills the capillary tube of the blood gas diffusion tube. The lower end of the diffusion tube is placed in a beaker of water and the stopper is

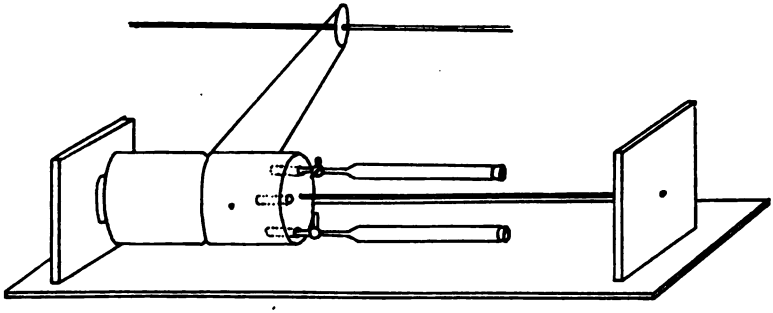


FIG. 2. Rotating apparatus.

withdrawn under water. The beaker is raised or lowered until the levels of the fluid inside and outside the tube are the same, and the volume of the air in the tube is read. The stop-cock at the top of the diffusion tube is then turned and a sample of the air within the tube is drawn over into the analyzer and analyzed for oxygen.

The residual gas from this analysis is nitrogen. As the percentages of oxygen and nitrogen in atmospheric air are known, it is easy to calculate from the volume of this residual nitrogen the exact amount of oxygen which would have been found by the analysis if no oxygen had been given off by the blood. To do this multiply the residual nitrogen by $\frac{20.93}{79.04}$, or 0.265. The volume of oxygen thus calculated to have been in the air is sub-

Digitized by Google

tracted from that found. A correction is then made for the volume of gas which remained in the diffusion tube and connections after the sample was drawn. Corrections are also made for barometric pressure and temperature, for the volume of gas is always expressed as it would be at 0°C. and 760 mm. barometer. The following is an example of this calculation.

Volume of air in diffusion tube	= 10.1 cc.	(1)
Volume taken for analysis	= 9.440 "	(2)
Nitrogen remaining after absorption of oxygen.	= 7.342 "	(3)
Oxygen absorbed, *i.e.*, (2)–(3)	= 2.098 "	(4)
Volume of atmospheric oxygen in volume of gas taken for analysis, *i.e.*, (3) $\times \frac{20.93}{79.04}$	= 1.945 "	(5)
Oxygen from blood in volume of gas analyzed, *i.e.* (4)–(5)	= 0.153 "	(6)
Total oxygen from blood, *i.e.*, (6) $\times \frac{(1)}{(2)}$	= 0.163 "	(7)
Oxygen reduced from barometric pressure 755 and temperature 20° to 760 and 0°, *i.e.*, (7) $\times \frac{760}{755} \times \frac{273}{(273+20)}$*	= 0.147 "	(8)
Volumes per cent oxygen in blood, *i.e.*, (8) $\times$ 100	= 14.7	

* Tables for these factors of pressure and temperature will be found in any handbook of chemical constants.

Comparative determinations of the oxygen content of the blood by the Barcroft-Haldane method using the Brodie apparatus (5) and by our method gave the following results.

Barcroft-Haldane method.	Gas analysis method.
16.2	16.3
16.5	16.1

The two methods are we believe of approximately the same order of accuracy. To one accustomed to the use of the gas analyzer, but not in practice for the use of the Barcroft-Haldane method in its usual form, our modification is, we believe, decidedly the easier.

The precision of the method may be increased,—at least theoretically—by filling the diffusion tube initially with nitrogen (from the analyzer) instead of air, inserting the stopper under

Digitized by Google

water, and injecting the blood with the hypodermic syringe. But this refinement takes time and requires extreme care to avoid admitting the least trace of air.

CO_2 Determination.

The CO_2 content of the blood is determined in exactly the same manner and with the same tube, merely using the tartaric

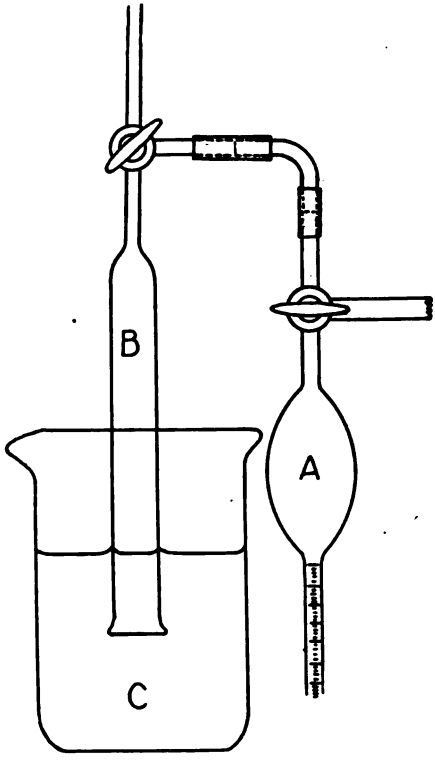


FIG. 3. A, gas burette of analyzer; B, diffusion tube; C, beaker of water.

acid solution instead of ferricyanide. (When the amount of gas in the blood is very high it is advisable to use only half quantities of blood and reagent; namely, 0.75 cc. of ammonia, 0.5 cc. of blood, and 0.10 cc. of tartaric acid.) Owing to the solubility of CO_2 it is necesary in the calculation of the results to take into account that part of the gas which remains in solution in the

Digitized by Google

acidified diluted blood. This correction for any temperature may be obtained from Curve 1 in Fig. 4.

A typical calculation of the results obtained is as follows.

Volume of air at 18°C. (compressed) in diffusion tube before removing stopper.....	= 10.9	cc.	(1)
Volume of air (at atmospheric pressure) in diffusion tube after removing stopper.....	= 11.1	"	(2)
Volume taken for analysis.....	= 7.774	"	(3)
Gas remaining after absorption of CO_2.....	= 7.506	"	(4)
CO_2 absorbed, *i.e.*, (3)–(4).....	= 0.268	"	(5)
Total CO_2 in air in tube, *i.e.*, (5) $\times \frac{(2)}{(3)}$.....	= 0.383	"	(6)
CO_2 in solution in acidified blood, *i.e.*, volume of liquid (2.75 cc.) $\times$ solubility coefficient (1.0) $\times$ CO_2 in air in tube $\frac{(6)}{(1)}$.....	= 0.096	"	(7)
Total CO_2 from blood, *i.e.*, (6) + (7).....	= 0.479	"	(8)
CO_2 reduced from barometric pressure 762 and temperature 26° to 760 and 0°, *i.e.*, (8) $\times \frac{760}{762} \cdot \times \frac{273}{(273+18)}$.....	= 0.448	"	(9)
Volumes per cent CO_2 in blood, *i.e.*, (9) $\times$ 100	= 44.8		

Comparison of the results obtained with the original Barcroft-Haldane method and with our modification shows in every case that the latter gives figures 2 to 3 volumes per cent higher than those obtained with the older method. This is due to the fact that the heavy precipitate produced by adding both ferricyanide and acid to blood renders it extremely difficult in the Brodie apparatus to shake the last trace of CO_2 out of the thick coagulum.

Simultaneous Determination of Oxygen and CO_2.

There appears no reason (except that just mentioned) why both gases should not be determined on a single sample of blood by first liberating the oxygen and then the CO_2. We have found that this can in fact be done (using half quantities of blood and reagents or in diffusion tubes of 20 cc. capacity) if a small glass rod or some lead shot is put into the tube to assist in breaking up the heavy masses of the precipitate formed by the addition

Digitized by Google

of the acid after the ferricyanide. Practically, however, unless the quantity of blood available is extremely limited it is easier and more accurate to make the two determinations separately. Duplicate determinations for both gases independently can be made by this method in half an hour.

Solubility of CO_2 in Acidified Blood Solutions.

As the correction for the amount of CO_2 which remains dissolved in the fluid is considerable we have devoted particular care

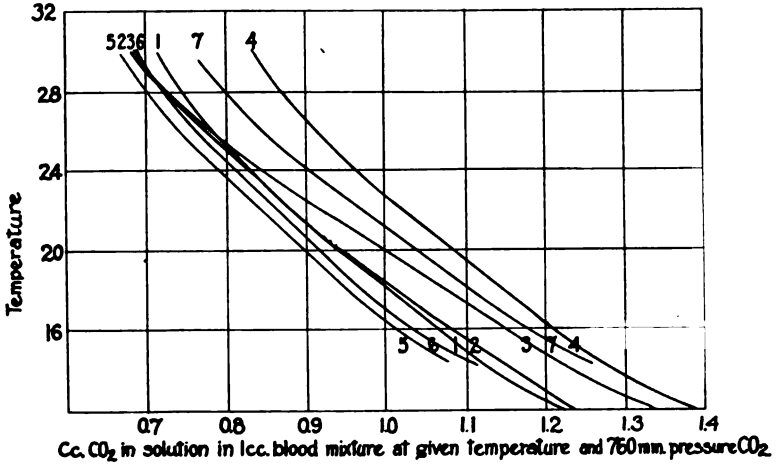


FIG. 4. Curves expressing solubility of CO_2.

The acid-blood mixture of Curves 1, 2, 3, 4, and 7 was made as follows: ammonia 1.5 cc., blood 1.0 cc., tartaric acid 0.25 cc. The mixture of Curves 5 and 6 was made as follows: ammonia 2.0 cc., blood 1.0 cc., tartaric acid 0.5 cc.

Curves 1 and 2, fresh human blood; Curve 3, fresh cat blood; Curve 4, blood of dog anesthetized with chloratone and alcohol; Curve 5, fresh pig blood; Curves 6 and 7, human serum.

to this matter. Various arrangements of apparatus were tried but finally one similar to that shown in Fig. 3 (except that the rubber tube connecting the diffusion tube and gas burette was 4 or 5 cm. long to allow shaking the tube) proved most convenient, as an entire series for all temperatures could be run from a single filling of the tube.

Digitized by Google

The method employed consisted in first filling the diffusion tube and burette with pure CO_2 gas from a Kipp generator. The rubber stopper was then inserted in the diffusion tube. The tube was immersed in water at various temperatures and the volume of gas read on the burette. 2 cc. of diluted acidified blood entirely freed from CO_2 were injected through the stopper into the diffusion tube. The tube was thoroughly shaken at various temperatures and the volume of gas going into solution was read from the burette. In the calculation allowance was made for the fact that the gas in the burette remained always at room temperature. The curves in Fig. 4 give the relative distribution of CO_2 at temperatures between 12° and 30°C. in gaseous form and in solution.

BIBLIOGRAPHY.

1. Barcroft, J., and Haldane, J. S., *J. Physiol.*, 1902, xxviii, 232.
2. Henderson, Y., and Morriss, W. H., *J. Biol. Chem.*, 1917, xxxi, 217.
3. Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 354.
4. Henderson, Y., *J. Biol. Chem.*, 1918, xxxiii, 32.
5. Brodie, T. G., *J. Physiol.*, 1909–10, xxxix, 391.

Digitized by Google

APPLICATIONS OF GAS ANALYSIS.

VI. THE RESPIRATORY EXCHANGE AND INDIRECT CALORIMETRY.

By YANDELL HENDERSON.

(*From the Physiological Laboratory, Yale Medical School, New Haven.*)

(Received for publication, October 30, 1917.)

The analysis of 10 cc. of expired air to determine its percentage of oxygen and CO_2 is not appreciably more difficult than the determination of the nitrogen in 10 cc. of urine by the Kjeldahl method. The nitrogen found in the sample multiplied by the total volume of the urine from which it was taken gives us the nitrogen metabolism of the body. In the same way the oxygen (deficit) and the CO_2 found in the sample of expired air multiplied by the total volume of the air expired in a given time afford the oxygen and carbon metabolism, and, by indirect calorimetry, the total energy exchange of the body.

Certainly a knowledge of the oxygen, carbon, and energy exchanges is quite as important in a wide range of problems as is the nitrogen exchange. Each should be equally a matter of routine in every well equipped biochemical and clinical laboratory. In fact however the means and habit of determining nitrogen are universal, while determinations of the respiratory exchange are confined to a very few highly specialized laboratories.

The reason for this anomaly lies in the fact that it is generally assumed that the elaborate and expensive apparatus of the "closed circuit" method used by Benedict, Lusk, and their collaborators is necessary. This is far from the truth. In fact, in a thorough comparative study and critique of all available methods Carpenter (1), although he does not say so explicitly, has shown implicitly that the (Tissot) "open circuit" method of collecting the expired air in a graduated spirometer and analyzing a sample is equal in accuracy and superior in simplicity to the closed circuit method.

Digitized by Google

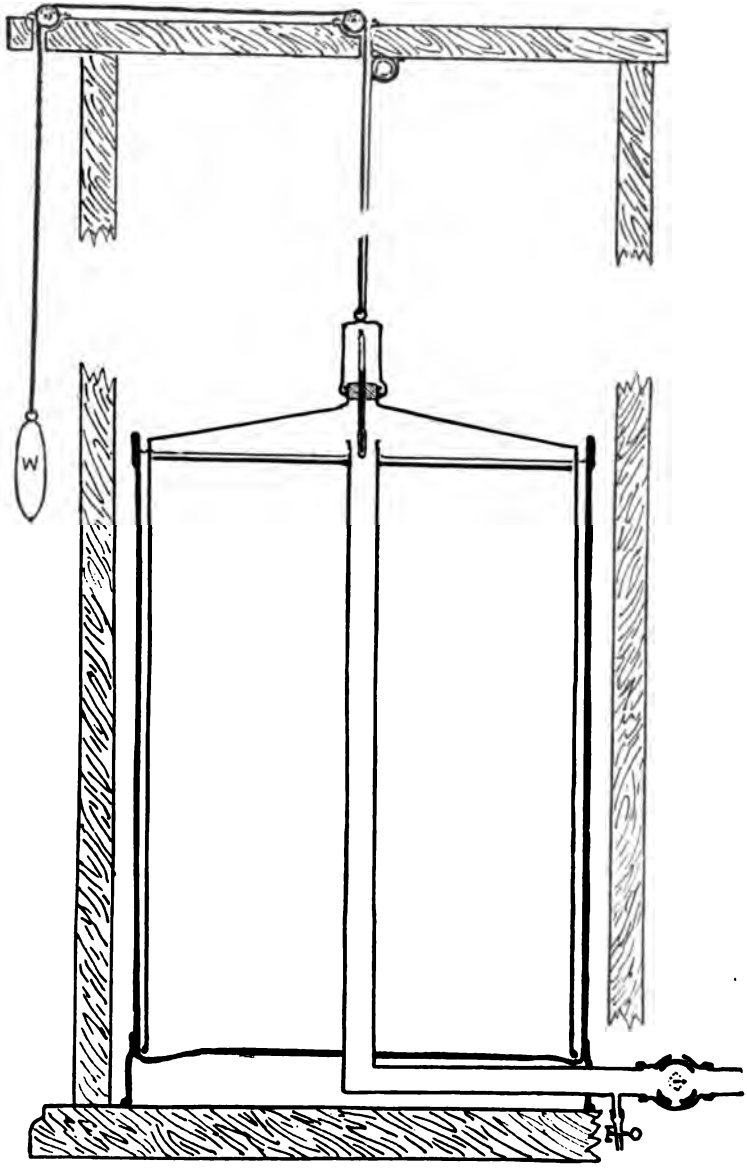


FIG. 1.

Digitized by Google

Apparatus for gas analysis has been discussed in the fourth paper of this series. In addition to a gas analyzer one needs a large graduated spirometer, a flexible tube, nose clip, and a mouthpiece with inspiratory and expiratory valves.

The spirometer shown in Fig. 1 is a simple form which has proved easily constructed and sufficiently accurate in use in this laboratory. The lower container consists of a common galvanized iron ash can. The tube is iron pipe (bore 1 inch) with a brass cock such as is used on steam-pipes. A round hole (¾ inch) is bored in the side so that it serves as a 3-way cock. A disk of galvanized iron is fastened near the top of the pipe and lies just below the surface of the water with which the can is filled. This prevents the diffusion of any considerable amount of CO_2 into the water. The bell or floating container is made of tin. It should be as nearly as possible of exactly the same diameter everywhere (this requirement is easily met by any competent tinsmith). The cord to the counterpoise weight runs over window sash pulleys. The rise of the bell of the gasometer is measured by a self-winding steel mm. tape measure of which the case is fastened to the cross bar of the wooden frame. The volume of the bell per mm. rise is merely its diameter multiplied by πr^2. The error which would occur if the dead space of the apparatus were filled with fresh air is eliminated by running a fore period in each experiment during which the gasometer is partly filled with expired air. Just before the period of the observation it is emptied through the side hole in the brass cock, thus leaving the dead space of the apparatus (tube and top of bell) filled with expired air.

To connect the mouthpiece and the spirometer the best tubing is the corrugated rubber used in mine rescue apparatus, but this is expensive and a very satisfactory substitute is afforded by a piece of vacuum cleaner tubing, which can be ordered in the proper length (2 or 3 feet) with heavy rubber nipples at the ends, from any vacuum cleaner shop. The standard rubber and metal mouthpiece has mica disk inspiratory and expiratory valves attached to it.[1]

A form of valve which has proved convenient has recently been devised at the University of Minnesota by Professors A. D. Hirschfelder and E. D. Brown. By their kind permission a diagram of two of these valves arranged for inspiration and expiration on a T-tube is shown in Fig. 2. Each valve is made of a large tin

[1] These valves on a metal mouthpiece, the rubber mouthpiece and corrugated tube and nose clip, as well as the Douglas bag with a large aluminum 3-way cock and other similar equipment are obtainable from H. N. Elmer, 1140 Monadnock Building, Chicago, the agent for Siebe, Gorman & Company of London.

Digitized by Google

salve box with a disk of sheet rubber inside, held in place by a ring of spring wire soldered to the box at one point. The crack round the box is made tight with adhesive plaster. In the figure the cover of one valve is removed to show the rubber and wire.

A double valve (inspiratory and expiratory) which also has been used in this laboratory is shown in Fig. 3. It is made of two pieces of brass tubing (1½ and 1 inch interior diameter respectively) arranged concentrically. The moving part of each valve is a strip of heavy sheet rubber inside the tube covering holes

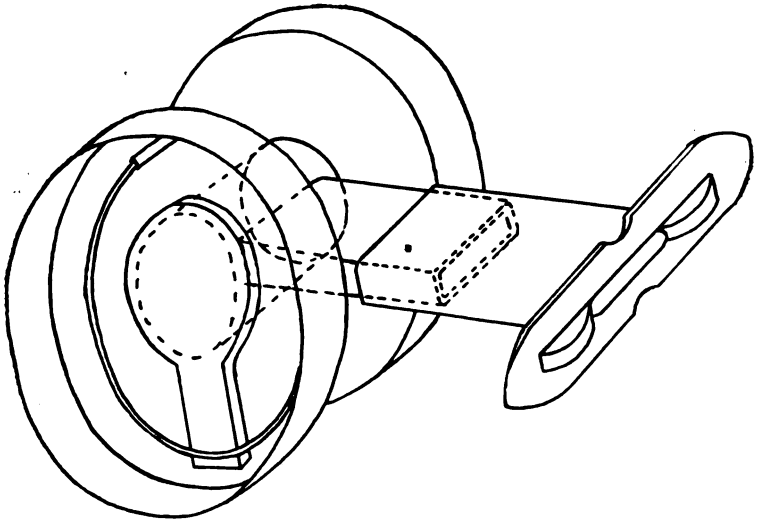


FIG. 2.

bored through the tubing and held in place by a metal clip near the middle of the strip. The lower ends of the tubes are closed with paraffined corks.

Perhaps no better idea can be given of the extensive use which can be made of the open circuit method combined with gas analysis than by reproducing here the directions given to the students in the routine course of physiology in this laboratory. These directions are typewritten together with the tables mentioned and are pasted upon pieces of compo board so that they can be conveniently carried about to the points where work is being done.

Digitized by Google

For the experiments in which the subject remains in one place the spirometer is used; for those in which he walks about the Douglas bag (2) is employed. The three tables referred to are as follows.

Table I.—For reduction to dry air at 0° and 760 mm. of mercury of 100 volumes of air saturated with moisture at different temperatures and pressures. This is reproduced from page 55 of Haldane's *Methods of Air Analysis* (3).

Table II.—For transforming apparent into true respiratory quotients. This is reproduced from page 57 of Haldane's *Methods of Air Analysis*. (The table includes one error; in the column of true respiratory quotients, 1.031 should be 1.131.)

Table III.—For the heat value of 1 liter of oxygen consumed in the body at various respiratory quotients. This is taken from page 357 of Volume xii of this *Journal* (4).

The directions are as follows.

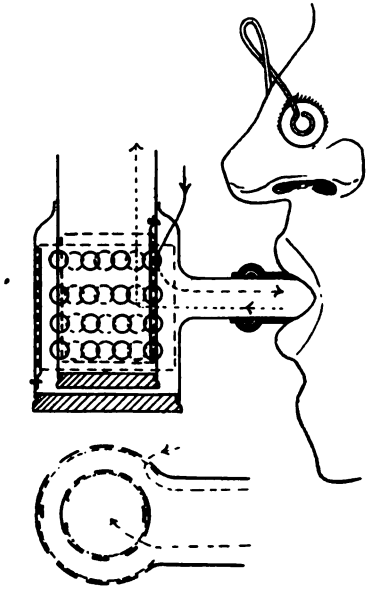


FIG. 3.

Digitized by Google

Calculation of Results of Experiments on Respiratory Exchange with Spirometer, Douglas Bag, and Small Animal Respiration Apparatus.

Analyze sample of air from the spirometer or bag for percentage of oxygen and CO_2. The volume of the gasometer is obtained by knowing the diameter of the bell, calculating its cross-section in sq. cm. by πr^2, and reading the distance the bell has risen on the self-winding steel tape measure fastened at the top of the bell. If the bag is used, measure contents of bag by passing the air through a gas meter and note temperature in the meter. Read barometer. By means of Table I reduce the volume of air expired in 1 minute to standard pressure and temperature (0°C. and 760 mm.).

From the CO_2 percentage subtract the CO_2 of the inspired air (0.03), and multiply by the (reduced) volume of air expired in 1 minute. This gives the CO_2 production per minute.

"The volume of oxygen absorbed is less easy to calculate however, as the volume of dry air has diminished in the process of respiration because more oxygen has been taken up than carbon dioxide has been given off. Since nitrogen is neither taken up nor given off in respiration, it is evident that for every 100 volumes of expired air there corresponded in the inspired air, not 20.93 volumes of oxygen, but $\frac{20.93}{79.04}$, or 0.2648, multiplied by the percentage of nitrogen in the expired air. By subtracting from the oxygen figure so obtained the oxygen percentage found by analysis, the true per cent of oxygen consumed is found. Multiplying the volume of air expired per minute by this percentage gives the oxygen really consumed, and dividing the CO_2 produced by the oxygen consumed gives the respiratory quotient" (Haldane, 3).

Instead of making this calculation, the results may be obtained by means of Table II. By this table the apparent respiratory quotients [CO_2 per cent found by analysis divided by (20.93 minus oxygen found by analysis)] can be transformed into true quotients, and the correct intake of oxygen obtained by dividing the output of carbon dioxide by the true respiratory quotient.

From Table III for indirect calorimetry the amount of energy produced and the character and amounts of the substances oxidized in the body are obtained by taking the figure corresponding to the respiratory quotient and multiplying it by the oxygen consumption.

To calculate the respiratory dead space (5) get the tidal volume (*i.e.*, total expired air divided by number of breaths) and multiply by

$$1.00 - \frac{CO_2 \text{ per cent in mixed expired air}}{CO_2 \text{ per cent in alveolar air}}.$$

The dead space for oxygen is obtained similarly. The oxygen pulse (6) is the oxygen consumed per minute divided by the pulse rate.

The external work on the stationary bicycle (7) equals the product of the resistance in kilos by the circumference of the wheel in meters by the number of revolutions per minute.

Digitized by Google

The object of this paper (the last of the series) has been to make more easily available facts and methods already known. The object of the entire series has been to emphasize the wide range of methods and problems for which gas analysis is a master key.

The expenses of all the investigations of this series of papers have been defrayed by the Loomis Medical Research Fund.

BIBLIOGRAPHY.

1. Carpenter, T. M., A comparison of methods for determining the respiratory exchange of man, *Carnegie Institution of Washington, Publication No. 216*, 1915.
2. Douglas, C. G., *J. Physiol.*, 1911, xlii, p. xvii.
3. Haldane, J. S., Methods of Air Analysis, London, 1912.
4. Williams, H. B., Riche, J. A., and Lusk, G., *J. Biol. Chem.*, 1912, xii, 357.
5. Henderson, Y., Chillingworth, F. P., and Whitney, J. L., *Am. J. Physiol.*, 1915, xxxviii, 1. Haldane, *ibid.*, 1915, xxxviii, 20.
6. Henderson, Y., and Prince, A. L., *Am. J. Physiol.*, 1914, xxxv, 106.
7. Martin, C. J., *J. Physiol.*, 1914, xlviii, p. xv.

Digitized by Google

Digitized by Google

A STUDY OF THE DIETARY ESSENTIAL, WATER-SOLUBLE B, IN RELATION TO ITS SOLUBILITY AND STABILITY TOWARDS REAGENTS.

By E. V. McCOLLUM and N. SIMMONDS.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, November 12, 1917.)

Since the discovery by Eijkman (1) of a means of producing an experimental polyneuritis in birds by feeding them a diet restricted to polished rice, and its relief by feeding rice polishings, interest in the problem of the isolation and identification of the curative substance has steadily increased (2). Without exception the methods which have been adopted for the isolation of the physiologically active substance have involved extracting it with either water or alcohol and subsequent precipitation with phosphotungstic acid, mercuric chloride, or silver nitrate and barium hydroxide. The former reagent has been employed extensively. The decomposition of the phosphotungstic precipitate by means of alkali such as barium hydroxide has been found to be attended by great loss of curative properties of the preparations. Better success has attended the use of barium acetate and of lead acetate for this decomposition (3).

The various modifications of the methods of separation, which were based upon precipitation with reagents, have yielded but meager results, and it seemed to us that prospects for success in the isolation of this interesting substance must be based upon an entirely different line of procedure from that employed in the past. It was with a view to the development of a new method for the separation of the physiologically indispensable substance which we term water-soluble B (4), and which we believe to be the specific complex, a lack of which causes the development of polyneuritis, that the experiments reported in this paper were conducted. The plan contemplates a complete study of the solubility of the water-soluble B in all organic solvents available.

Digitized by Google

We sought, as suitable material with which to work, a vegetable product which, after the removal of fats by ether, gave promise of yielding the smallest amount of alcohol-soluble matter. The navy bean was selected, but this seed we now feel will not prove superior to a number of others which might have been employed. The bean is extremely difficult to grind to a fine state and after ether has removed everything which it is capable of extracting, alcohol still extracts about 4 per cent of the original weight of the bean. The bean is, however, very rich in the dietary B (5), and, as will be shown later, there seems good reason to expect that a method can be developed for separating the desired substance in a greatly concentrated form.

The navy beans were ground and dried, then exhausted with ether. This solvent does not take up a demonstrable amount of the water-soluble B. The ether-extracted material was subsequently extracted as described in the experimental part of this paper. Wheat embryo similarly treated was employed in some of the experiments.

We believe our method which consists of testing preparations for the presence of the antineuritic substance, water-soluble B, by relieving the polyneuritic condition and inducing subsequent growth in a mammal, to be greatly superior to the conventional method of "curing" pigeons which have been fed an exclusive diet of polished rice until they develop the disease. Polished rice is very deficient from the dietary standpoint in certain mineral salts, the fat-soluble A, and protein in addition to the specific substance water-soluble B, the lack of which leads to the development of polyneuritis (6). Recovery cannot be permanent on such a diet, and the observation of the "curative" effects of the administered substances is necessarily limited to a brief period.

We decided to adopt the rat instead of the pigeon as an experimental animal, because of the fact that the element of growth can be included in the observations. Where growth takes place and is sustained, there can be no possible doubt that we are supplying a chemical complex which is necessary as a *food* rather than a complex which produces its effects through pharmacological action. In the work with pigeons, previously reported (4), we had ample experience to convince us of the prompt recovery of birds from the polyneuritic state when given various

Digitized by Google

preparations. Careful reading of the literature relating to studies on polyneuritis in birds revealed so many confusing statements that we decided to attempt to perfect the technique for employing the rat for such studies. We feel convinced that a satisfactory procedure has been developed.

Funk (7) says that when the curative fraction obtained from either dry yeast or rice polishings was administered orally or subcutaneously to beri-beri pigeons, "the animals recovered very speedily, often in 2–3 hrs., but it was found impossible to keep them permanently on polished rice even when injections were repeated every few days."

Williams (8) has pointed out, and our experience harmonizes with his, that pigeons restricted to a diet of polished rice do not all run the same course. Many never develop acute polyneuritis, but waste away and die of starvation. Such birds are of no value in experimental work. Some, he states, recover temporarily from an acute attack without any treatment.

Eijkman (9) injected doses of 20 to 40 mg. of a mixture of one part NaCl and three parts KCl into chickens and pigeons and observed cures in pigeons but not in chickens. These results seem to justify the belief that temporary relief from paresis is less satisfactory as a test for the physiologically indispensable substance water-soluble B than is our method in which the diet is properly planned so as to be satisfactory for growth except for the absence of the one unidentified substance. With our diet resumption of growth actually takes place on the addition of the water-soluble B to the diet.

Antineuritic properties have been attributed to nicotinic acid (10) and to adenine (11), betaine, allantoin, and certain pyrimidines (12), and Williams has observed curative action in a certain isomer of α-hydroxypyridine (8). He has attempted to account for all of these observations by assuming that the needs of the animal are for a specific type of labile isomerism rather than a specific chemical complex as in the case of certain amino-acids in their relation to protein metabolism. That this explanation should be the correct one we believe improbable. The antineuritic substance employed by Williams was relatively labile, and passed easily into a form which possessed no curative properties. In the natural foods the evidence available from well

Digitized by Google

controlled experiments all indicates that the dietary essential termed by us the water-soluble B is not readily destroyed by aging, exposure to the atmosphere, or by heat at 100–112°C. (5) in neutral reaction. The experience of all investigators who have employed precipitation methods for the concentration of this substance indicates that it is very stable in dilute acid solutions. Indeed the statement has been made by Vedder and Williams (13) and by Funk that hydrolyzed preparations were more active than before hydrolysis.

It is possible to suggest an alternative explanation for Williams' hypothesis which will account for the occasional relief of polyneuritic pigeons by such a list of unrelated substances as nicotinic acid, certain pyrimidines, certain purines, and certain hydroxypyridines. The following explanation, while purely speculative, may possibly account for the recorded observations and assist in clarifying the confusing data relating to this subject.

Histological methods have shown that in polyneuritic animals there is a degeneration of the motor cells of the cord. This change is progressive; some cells present the normal appearance while others in the same field are degenerated. It would appear plausible that when the motor cell changes have reached a certain point loss of function supervenes and paralysis results. There still remain in the cord of a paralyzed animal motor cells which appear normal when stained and which may be capable of restoring the motor functions of the muscles when influenced by substances which stimulate them to heightened sensitiveness. In other words, the temporary relief of polyneuritis may be the result of the pharmacological action of certain substances rather than a response with renewed function of cells which have been subjected to a selective fast and later have been supplied with the missing food complex. If this line of reasoning could be shown to be valid, it would follow that experiments with pure chemical substances of known constitution, with a view to finding by good fortune the one playing an important physiological rôle, might be entirely misleading *unless it were shown that the "cure" was permanent.* For such complete proof it is necessary to demonstrate the resumption of growth and maintenance of health as long as the substance is supplied in the food mixture. Animals which have been brought into a critical condition where death is

Digitized by Google

certain within 24 hours, and which have been permanently cured on the addition to the food mixture of a very small amount of a preparation obtained in efforts to isolate the unidentified food factor, furnish absolute proof that the physiologically active *dietary factor* is being dealt with. Sustained normal function is indispensable to adequate proof that the dietary essential in question is being administered. If this condition should be insisted upon by investigators before concluding that a test is positive, it is probable that we should not have such a list of totally unrelated chemical substances reported as protective against polyneuritis.

Method of Experimentation.

When young rats are placed upon a diet which is satisfactory in every respect except for the absence of either of the dietary factors, fat-soluble A or water-soluble B, a considerable gain in weight may take place during the first 3 weeks. This does not always happen, but with vigorous rats it is of frequent occurrence. After the 2nd or 3rd week, on the experimental diet, there is no further increase in weight and either a brief period of maintenance is followed by rapid decline or steady loss of weight begins and death follows after a variable period, unless the missing dietary essential is supplied. Loss of hair and feebleness are seen with either type of deficient diet.

When the diet is lacking in the water-soluble B, but is properly constituted in other respects, typical polyneuritis results in many of the experimental animals. Funk (7) holds the view that beri-beri has not been produced in animals other than man and birds, and he believes that only those species whose end-product of purine metabolism is uric acid can develop the disease. The rat in common with other mammals with the exception of man, the higher apes, and the curious exception of the Dalmatian dog recently observed by Benedict (14), excretes allantoin, and according to Funk's classification should not suffer from this disease. In our experience rats restricted to a diet which is in every way adequate except for the absence of the antineuritic substance, water-soluble B, suffer decline in weight and loss of muscular control, especially in the hind limbs, and die within a few hours unless the necessary complex is supplied. Histological

Digitized by Google

studies have shown that there is degeneration of the motor cells of the cord in animals restricted to such a diet. We see, therefore, reason to believe that our animals in the experiments described are actually in the polyneuritic state. When kept upon a diet of polished rice, young rats do not live long enough to become paralyzed owing to the deficiency of *polished* rice in respect to protein, inorganic elements, and both the A and B (6). Rats suffering from polyneuritis are promptly relieved by the administration of the water-soluble unknown. When young rats are fed the diet of purified foodstuffs, plus butter fat, as employed in this work, polyneuritis occurs ordinarily between the 30th to the 40th day.

Animals given a diet which is properly constituted except that it is lacking in the fat-soluble A, may increase in weight during the first 2 weeks, then they gradually lose weight, become emaciated, and suffer from edema of the eyelids (15). We have recently (16) described this condition as xerophthalmia and believe it is due to this specific dietary deficiency. There is no loss of motor function in these animals. Their eyes are very much irritated and they scratch them and thereby cause mechanical injury. Recovery is prompt when the missing dietary factor is supplied, otherwise permanent blindness ensues and death supervenes, usually within 3 months.

Since we have no chemical methods for the detection of either of these two unidentified dietary essentials we must employ biological tests as a guide to their isolation. The period required in order to make the test complete must extend over several weeks because of the ability of the more vigorous animals to grow to a slight extent during the first 2 or 3 weeks, even when the substance to be tested for is absent from the diet. Preparations made with a view to separating the factor B in a pure form frequently led, if fed from the beginning of the experiment, to long maintenance and confusing data. This is due, on the one hand, to the difficulty, if not impossibility, of making complete extractions, so that the residues still contain traces of the factor B, and, on the other hand, to the fact that in all probability solvents in which this substance is regarded as insoluble remove small amounts when the extractions are greatly prolonged. Such residual amounts may well be wholly inadequate to cause recovery of

Digitized by Google

an animal in a serious pathological state, but, when furnished from the beginning of the feeding experiment, may extend the period of growth or maintenance and delay the appearance of signs of failure.

In conducting the work reported in this paper, we followed the plan of feeding a diet of purified food substances together with 5 per cent of butter fat to supply an abundance of the fat-soluble A. This diet was complete except that it was free from the water-soluble B. The rats were confined to this food mixture until they either had become stationary in weight or were declining. By the 5th week nearly all were either stationary in weight or were failing and they almost invariably showed signs of paralysis at about this time. When the rats were thus prepared, the material to be tested for the water-soluble B was put into the diet. The animals then either continued to decline or responded with growth. This method served to show within 2 weeks whether the substance B in significant amount was in the preparation under investigation. This procedure makes the test decisive. It also greatly shortens the time required to make the test.

The scheme of applying successively to *raw* navy beans the solvents ether, benzene, and 95 per cent alcohol, in the order named, and of feeding in separate experiments the extract and residue in the case of benzene and alcohol showed that benzene does not remove the water-soluble B from ether-extracted beans while hot alcohol does. The extraction with hot alcohol is not complete in a Soxhlet apparatus in 18 hours.

A surprising result was observed when the material which was dissolved from beans by hot alcohol was deposited on dextrin and the latter then extracted with hot benzene. The *benzene-soluble* material obtained by this procedure was very effective in inducing growth and in causing the prompt recovery of rats in the polyneuritic state. A benzene extract prepared directly from ether-extracted beans, however, does not contain an appreciable amount of the substance B. Benzene, therefore, does not remove the water-soluble B from beans directly, but once this substance is extracted by alcohol it is soluble in benzene.

Acetone does not extract the water-soluble B when the solvent is applied directly to the ether-extracted beans, and extracts only traces when applied to the alcohol-soluble matter from beans.

Digitized by Google

It is, therefore, possible to exhaust beans successively with ether, benzene, and acetone, and then to extract the physiologically active substance with alcohol. If the alcohol is then evaporated upon some substance, as dextrin, to distribute the alcohol-soluble material over a large surface, the desired substance can then be extracted with benzene (17). It is obvious that by this procedure and with the introduction of still other solvents the substances which accompany the physiologically active compound can in great measure be eliminated. By the introduction of precipitation methods at that point where interfering substances have been eliminated as far as possible, the chances of success in the preparation of a pure product or the preparation of derivatives, and with a minimum of loss in manipulation, are much greater, we believe, than in the methods of procedure hitherto employed by others.

In the experimental part of this paper it is shown that the water-soluble B is not extracted directly from beans, wheat germ, or pig kidney by ether, benzene, or acetone, but is readily extracted in great part by alcohol. After being removed by alcohol it is shown to be soluble in benzene, but very slightly soluble in acetone. The probability that there should be two or more physiologically indispensable substances in what we term water-soluble B, both or all of which should show the same solubility relations with three solvents, is relatively small and lends support to our view that the substance which protects animals against polyneuritis is the only essential complex in the extracts described. In other words, the data support the view that there are no specific substances present in these extracts which protect against such diseases as scurvy, rickets, pellagra, sprue, etc., and tend to confirm our contention that the latter are not due to specific starvation as is the case with beri-beri and xerophthalmia (16).

In interpreting the charts it should be remembered that at the point marking the beginning of the second period the animals were at or near the point of showing loss of muscular control, and that response with even a slow rate of growth on the addition of a preparation is conclusive evidence of the presence of the physiologically active substance, water-soluble B.[1]

[1] Credit is due to Mr. H. Steenbock for the preparation of the extracts employed in this work.

Digitized by Google

In the following charts, the diet in *Period 1* consisted of purified foodstuffs plus 5 per cent of butter fat.

Casein	18.0
Salt mixture 185	3.7
Agar-agar	2.0
Dextrin	71.3
Butter fat	5.0

Composition of Salt Mixture 185.

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$Na\ H_2PO_4.H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Fe citrate	0.118
Ca lactate	1.300

Chart 1.[2] *Lots 953 A, 952 A, and 952 B.*

Lot 953 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the benzene extract of ether-extracted *raw* navy beans equivalent to 30 gm. of beans per 100 gm. of ration.

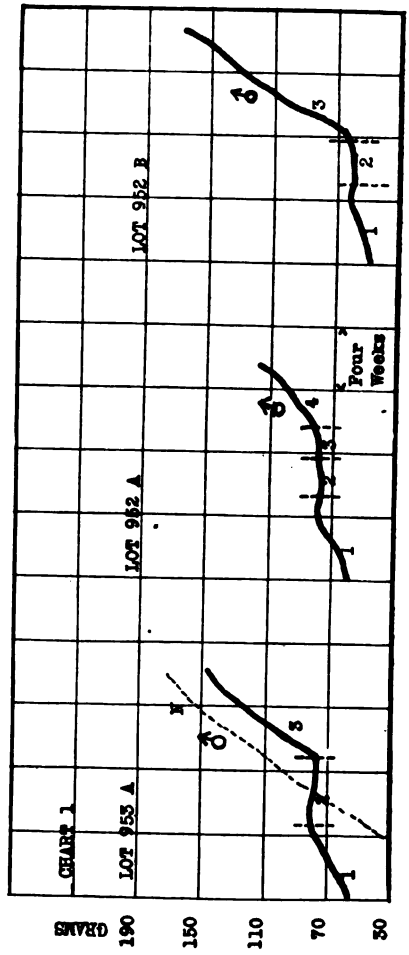
Period 3.—Ration: Same as Period 1 plus the benzene extract of the alcohol-soluble part of ether-extracted *raw* beans, equivalent to 30 gm. of beans per 100 gm. of ration; *i.e.*, alcohol-soluble,[3] benzene-soluble part of the bean.

This experiment shows that the water-soluble B is extracted from *raw* beans by hot alcohol, but not by ether or warm benzene. After the substance has been extracted from beans by means of alcohol, it is, however, soluble in benzene (see Chart 2, Lot 953 B, Period 4).

[2] The curves presented in this and the following charts are actual records of animals, and are representative of a group usually of four individuals. For economy of space a single record is shown. The broken curves marked N represent the normal expectation of growth.

[3] Only 95 per cent alcohol was employed in making the alcoholic extracts used in these experiments.

Digitized by Google



Digitized by Google

Lot 952 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the alcohol-soluble portion of ether-extracted *raw* navy beans evaporated on dextrin and extracted with acetone; *i.e.*, alcohol-soluble, acetone-insoluble part of beans. The ration contained the equivalent of 30 per cent of beans.

Period 3.—Ration: Same as Period 2 but with the alcohol-soluble, acetone-insoluble part of the *raw* beans heated 40 minutes at 15 pounds' pressure in an autoclave.

Period 4.—Ration: Same as Period 1 plus the acetone extract of the alcoholic extract of ether-extracted *raw* beans, plus the acetone-insoluble residue, *i.e.*, the equivalent of the total alcoholic extract, after being heated with acetone. The ration contained the equivalent of 30 per cent of beans (see preparation 951 B, under Chart 3).

In Chart 4, Lot 951 B, it is shown that growth could not be secured with the acetone-soluble part of the alcoholic extract of 30 per cent of *raw* beans in the diet. Since 10 per cent of beans supplies enough of the water-soluble B to induce growth (Chart 10, Lot 933) and neither the acetone extract or residue from the alcoholic extract of 30 per cent of beans in Lot 952 A, Chart 1 and Lot 951 B, Chart 4, induced growth, it seems certain that there is a slow destruction of the physiologically active substance in hot acetone.

Lot 952 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 25 per cent of *raw* beans extracted with ether, then with benzene.

Period 3.—Ration: Same as Period 2, but with the beans moistened with distilled water and heated at 15 pounds' pressure for 40 minutes in an autoclave.

Neither ether nor benzene removes the water-soluble B from beans. In the second period the rats apparently did not grow because of failure to eat the ration containing raw beans. After heating the bean residue with water and thus making it more palatable, growth at once took place, which shows that the factor B was still in the residue.

Preparation of Extract and Residue Used with Lots 953 A and 952 B.—1,000 gm. of finely ground raw navy beans were extracted continuously for 25 hours with alcohol-free ether. The residue was then extracted with benzene in three successive 6

Digitized by Google

hour periods using fresh solvent each time to reduce the amount of exposure to heat. 1.635 gm. of material were extracted in the first two 6 hour periods and 0.222 gm. was extracted in the last. They were made up to 100 gm. with dried dextrin. The residue was freed from benzene and later heated in the autoclave. The benzene-soluble part was employed in the ration of Lot 953 A, Period 2. The benzene-insoluble part was used with Lot 952 B, Period 2, and, after heating, in Period 3. For the alcohol-soluble, acetone-insoluble fraction, employed in Chart 1, Lot 952 A, Period 2, see under 951 A, Chart 3.

Preparation of the Extract and Residue Employed with Lots 953 A, Period 3, and 953 B, Period 2.—1,000 gm. of finely ground *raw* navy beans were extracted for 25 hours with alcohol-free ether. 13.33 gm. of material were thus removed. The extracted residue freed from ether was then extracted with 95 per cent alcohol in three successive 6 hour periods. After distilling off the alcohol 30.45 gm. of extractives were left from the first two 6 hour periods and 9.7 gm. from the last. They were united in alcohol-water solution and evaporated on dextrin. Final weight of dry material, 165.1 gm.

The dextrin which carried the alcoholic extracts was transferred to a small Soxhlet apparatus and extracted with benzene successively in a 6, 6¾, and an 8 hour period. The extract from the first two periods weighed 25.39 gm., from the last period 0.61 gm. They were united, dried at 37°C., and ground up without evaporating on dextrin. This was fed to Lot 953 A, Period 3, Chart 1, and to Lot 953 B, Period 4, Chart 2.

The residue from the benzene extraction (alcohol-soluble, benzene-insoluble material) was made up to 200 gm. with dextrin. This preparation was fed to Lot 953 B, Period 2, Chart 2. It was later moistened with distilled water and heated 40 minutes in an autoclave at 15 pounds' pressure.

Chart 2. Lots 953 B, 994 A, and 994 B.

Lot 953 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

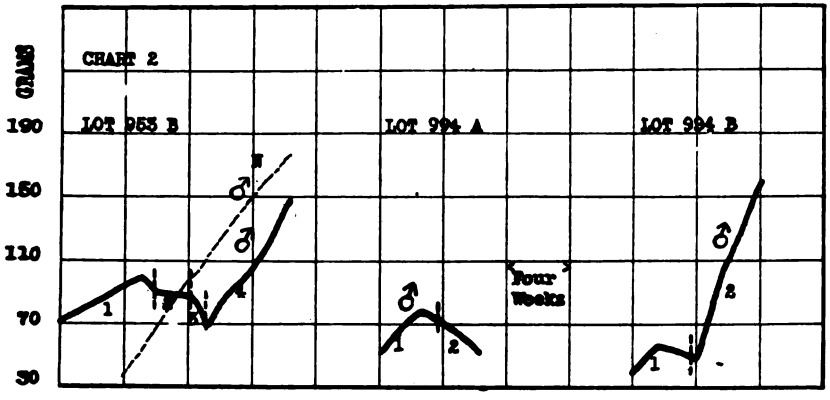
Period 2.—Ration: Same as Period 1 plus the portion of *raw* beans, soluble in alcohol but insoluble in benzene, equivalent to 30 per cent of beans.

Digitized by Google

Period 3.—Ration: Same as Period 2 but with alcohol-soluble, benzene-insoluble residue of *raw* beans heated 40 minutes in an autoclave at 15 pounds' pressure.

Period 4.—Ration: Same as Period 3 but with the benzene-soluble portion of the alcohol-soluble part of the beans included; *i.e.*, the total alcoholic extract of beans after treatment with benzene (used extract equivalent to 30 per cent of beans).

The alcoholic extract of *raw* navy beans contains the dietary essential water-soluble B. Although benzene does not extract this substance from the beans directly (Chart 1, Lot 953 A, Period 2; Chart 2, Lot 994 B), once it has been removed from the beans by means of hot alcohol, and the alcohol evaporated,



benzene dissolves the physiologically active substance fairly readily (Chart 1, Lot 953 A, Period 3; Chart 2, Lot 953 B, Period 4).

Lot 994 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the benzene extract of ether-extracted *cooked* navy beans, equivalent to 30 per cent of beans in the diet.

Benzene does not take out the water-soluble B from the beans directly (Chart 1, Lot 953 A, Period 2). It must be first removed by hot alcohol, after which it is fairly soluble in hot benzene (compare Chart 1, Lot 953 A, Periods 2 and 3, and Chart 2, Lot 953 B, Period 4). Lot 994 B, Period 2, shows that the

Digitized by Google

factor B is still in the residue of the *cooked* beans after extraction with ether and then with benzene.

Lot 994 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with 25 per cent of navy beans (*cooked*) extracted with both ether and benzene.

This experiment shows that in Chart 2, Lot 994 A, Period 2, benzene failed to take out the water-soluble B from *cooked* beans, although the substance was still present after the thorough cooking. Lot 994 B shows that the residue of beans after extraction with ether and then with benzene still contains the unknown B. As we pointed out under Chart 1, Lot 953 A, Period 3, the substance is soluble in benzene after first being removed by alcohol.

Preparation of Extract and Residue Used with Lots 994 A and 994 B.—1,161 gm. of *cooked* navy beans were extracted with alcohol-free ether for 18 hours. The residue was then extracted with benzene in three consecutive 6 hour periods. The first two 6 hour periods gave 5.36 gm. of extract, the last period gave 0.904 gm. of extract. The extracts were taken up in benzene, in which they readily redissolved, and then were evaporated on dextrin and made up to 77.4 gm. The extract (benzene-soluble material) was fed in the ration of Lot 994 A, Period 2. The residue (benzene-insoluble) was fed in the ration of Lot 994 B, Period 2.

Chart 3. Lots 954 A, 954 B, and 951 A.

Lot 954 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 25 per cent of the residue of *raw* navy beans after extraction with ether and then with acetone.

Period 3.—Ration: Same as Period 2 but with the bean residue moistened with distilled water and heated 40 minutes in an autoclave at 15 pounds' pressure.

Neither ether nor acetone removes the water-soluble B from raw beans (Chart 3, Lot 954 B, Period 2). There was no growth in Period 2 of Lot 954 A in which ether- and acetone-extracted *raw* beans were added to the diet, but this we attribute to failure of food consumption because in Period 3, after cooking the bean residue, which made it more palatable, rapid growth took place.

Digitized by Google

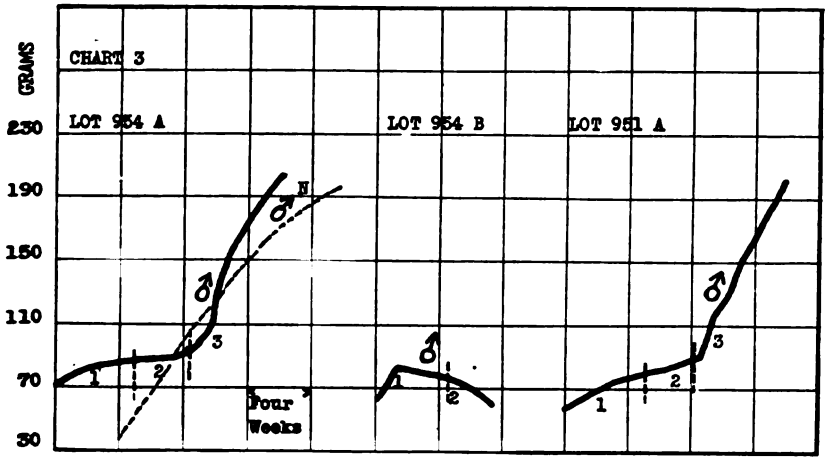
Lot 954 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 with an acetone extract of ether-extracted *raw* navy beans equivalent to 30 per cent of beans in the diet.

Acetone does not readily extract the water-soluble B from *raw* beans (see Chart 4, Lot 1,001 B, Period 2). The acetone-soluble part of the bean does not induce appreciable growth, but the acetone-insoluble *cooked* residue does (Chart 3, Lot 954 A, Period 3).

Lot 951 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with 25 per cent of the residue of *raw* navy beans after extraction with ether, then with alcohol.



Period 3.—Ration: Same as Period 2, but with the bean residue moistened with distilled water and heated in an autoclave 40 minutes at 15 pounds' pressure.

This experiment shows that the method of alcoholic extraction employed did not remove all of the water-soluble B from the beans. The residue was still potent in promoting growth.

Preparation of Extract and Residue Used with Lots 954 A, Period 2, and 954 B, Period 2.—1,000 gm. of finely ground *raw* navy beans were extracted continuously with alcohol-free ether for 24½ hours. 13.19 gm. of material were extracted. The residue was freed from ether at room temperature and extracted with

Digitized by Google

acetone in three consecutive 6 hour periods changing the flask containing the extract each time. On distilling off the acetone, 3.77 gm. were found to have been removed by extraction in the first two 6 hour periods and 0.75 gm. in the last. They were taken up in a small amount of ether and evaporated on dextrin (95.5 gm.) giving a final weight of 100 gm. This extract was fed in the ration of Lot 954 B, Period 2. The residue (that which was insoluble in acetone) was later heated and fed in the ration of Lot 954 A, Periods 2 and 3.

Preparation of Extract and Residue Used in Chart 1, Lot 952 A, Period 2, Chart 3, Lot 951 A, Period 2, and Chart 4, Lot 951 B, Period 2.—1,000 gm. of finely ground *raw* navy beans were extracted with alcohol-free ether for 25 hours. Upon evaporating off the ether, 13.30 gm. of material were found to have been removed by the extraction.

The residue from the above extraction was freed from ether at room temperature and then extracted with 95 per cent alcohol in three consecutive 6 hour periods, using fresh or recovered solvent each time in order to minimize the possible destructive action of heat on the extract. 25.5 gm. of material were left as the extract of the first two 6 hour periods after distilling off the alcohol and 7.85 gm. from the last 6 hour period. These extracts were taken up in a little water and alcohol and then evaporated on dextrin and dried at 90–95° for a few hours. The dry weight was 128.35 gm. The residue from the extraction was freed from alcohol by heating at 90° for 3 hours. Later part of this residue was soaked in water and heated in the autoclave at 15 pounds' pressure for 40 minutes. This preparation (alcohol-insoluble) was fed to Lot 951 A, Chart 3, Period 2.

The 128.35 gm. of dried dextrin residue carrying the alcoholic extract from 1,000 gm. of *raw* navy beans was next extracted with acetone in a small Soxhlet apparatus, again in three consecutive 6 hour periods. After removing the acetone with minimum heat exposure, 7.96 gm. of extract were left from the first two 6 hour periods and 0.81 gm. from the last 6 hour period. They were taken up in alcohol, evaporated on dextrin, and made up to 100 gm. This mixture was fed to Lot 951 B, Chart 4, Period 2. The residue from the acetone extraction was freed from acetone and made up to 125 gm. with dextrin. It was employed in the ration of Lot 952 A, Chart 1, Period 2.

Digitized by Google

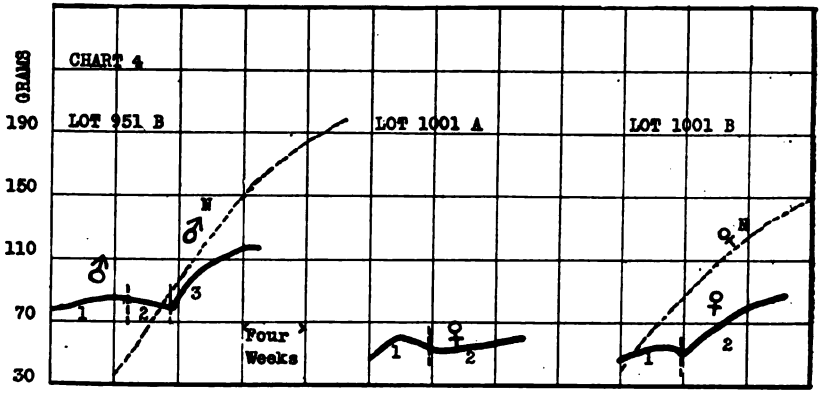
Chart 4. Lots 951 B, 1,001 A, and 1,001 B.

Lot 951 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the acetone-soluble part of the alcohol-soluble part of ether-extracted *raw* navy beans, equivalent to 30 per cent of beans in the diet.

Period 3.—Ration: Same as Period 2 but with the addition of the acetone-insoluble part of the alcohol-soluble part of beans, equivalent to 30 per cent of beans; *i.e.*, the equivalent of the entire alcohol-soluble part of the beans, after being heated with acetone.

This experiment shows that even after extraction with alcohol, the water-soluble B is but slightly soluble in acetone. This conclusion is further supported by Chart 4, Lot 1,001 B, Period 2, and Chart 5, 1,001 C, Period 2.



In Chart 1, Lot 952 A, Period 2, it is shown that no growth could be secured with the alcohol-soluble, acetone-insoluble portion of beans equivalent to 30 per cent of beans in the diet. This suggests that there is a slow destruction of the water-soluble B during heating with acetone.

Lot 1,001 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the acetone extract of the alcoholic extract and residue of same, of 10 gm. of ether-extracted *cooked* navy beans; *i.e.*, the equivalent of the alcoholic extract of navy beans after being heated with acetone.

Lot 1,001 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Digitized by Google

Period 2.—Ration: Same as Period 1 plus the acetone-soluble part of the alcoholic extract of ether-extracted *cooked* navy beans equivalent to 60 gm. of beans.

This experiment shows that the water-soluble B is slightly soluble in acetone but very much less so than in benzene (compare with Chart 1, Lot 953 A, Period 3, and Chart 5, Lot 1,001 C, Period 2).

Preparation of Extract and Residue Employed with Lots 1,001 A, 1,001 B, and 1,001 C.—935 gm. of *cooked* navy beans were extracted with ether for 18 hours. The residue, freed from ether, was then extracted with alcohol for 18 hours in three consecutive 6 hour periods, changing the flask containing the extract at the end of each period. The first 12 hours' extract evaporated on dextrin gave 26.7 gm. of material, the last 6 hours' gave 7.6 gm. The extracts were united and made up to 200 gm. with dextrin.

100 gm. of the above preparation equivalent to 467 gm. of beans were extracted with freshly redistilled acetone, as outlined above. The first 12 hours' extraction gave 14.7 gm. of extract, the last 6 hours' 0.5 gm. The extracts were united and made up with dextrin to 100 gm. This preparation was employed with Lot 1,001 B, Period 2. The residue was likewise made up to 100 gm. after the extraction. This preparation was employed with Chart 5, Lot 1,001 C, Period 2. Both the extract and residue were employed in the ration of Lot 1,001 A, Period 2.

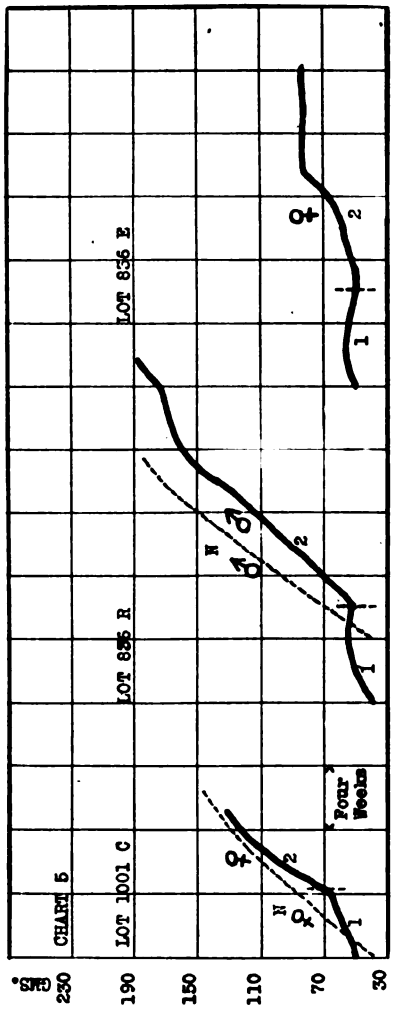
Chart 5. Lots 1,001 C, 836 R, and 836 E.

Lot 1,001 C. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the residue of the acetone extract of the alcoholic extract of ether-extracted *cooked* navy beans equivalent to 60 per cent of beans; *i.e.*, the alcohol-soluble, acetone-insoluble part.

This experiment shows that in time hot acetone separates a part of the water-soluble B from the *cooked* navy beans (see Chart 4, Lot 1,001 B), but leaves the larger part of it in the residue. Rats grow on both the acetone extract and its residue when the plane of intake is sufficiently raised.

Digitized by Google



Digitized by Google

Lot 836 R. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 3 per cent of the residue after ether and acetone extraction of wheat embryo.

This experiment shows that acetone does not take out to any appreciable extent the water-soluble B from ether-extracted wheat embryo. Studies with pigeons indicate that traces are removed by this solvent, for the acetone extract of wheat embryo relieves polyneuritic pigeons (4).

Lot 836 E. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the acetone extract of ether-extracted wheat embryo equivalent to 14.28 per cent of wheat embryo in the diet.

This experiment shows that acetone takes out the water-soluble B from wheat embryo to a very slight extent.

Preparation of the Extract and Residue Employed with Chart 5, Lots 836 R and 836 E; Chart 6, Lot 836 R + E.—500 gm. of wheat embryo previously extracted with alcohol-free ether for 18 hours were extracted continuously with hot acetone in a Soxhlet apparatus for 18 hours. The acetone was distilled off, the residue taken up with hot water, and evaporated on 300 gm. of dry dextrin. After drying, 3.2 gm. were found to have been removed in the extraction process. The extract was made up to 400 gm. with dextrin.

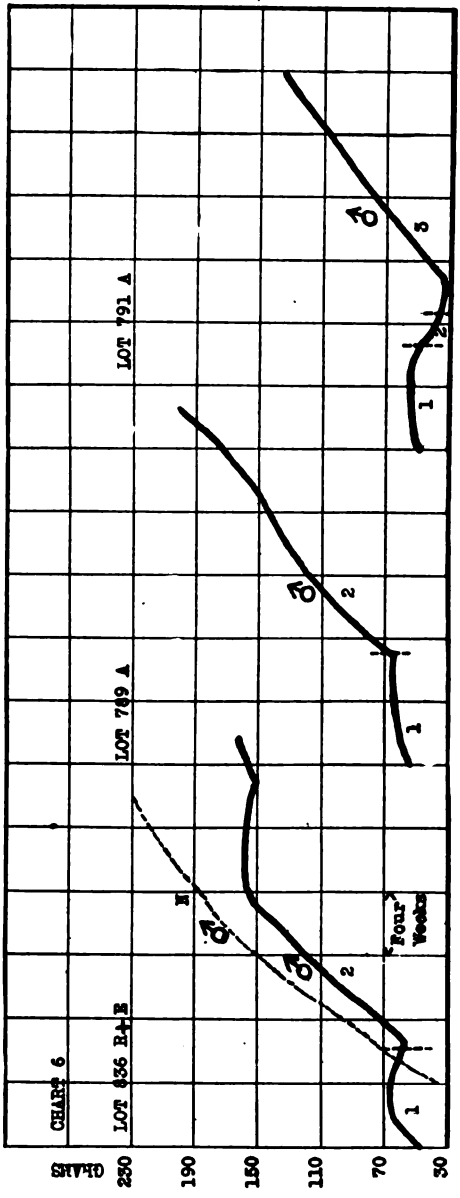
Chart 6. Lots 836 R + E, 789 A, and 791 A.

Lot 836 R + E. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the addition of 3 per cent of the ether- and acetone-extracted residue plus the acetone extract of 14 gm. of ether-extracted wheat embryo per 100 gm. of ration.

This experiment shows that the water-soluble B is not rapidly destroyed by prolonged treatment with hot acetone. There was no evidence from the appearance or activity that these rats receiving both the acetone extract and residue as in 836 R + E were in better condition than those receiving only the residue, as in Chart 5, Lot 836 R. This supports the view that there is but a *single substance* in what we term water-soluble B.

Digitized by Google



Digitized by Google

Lot 789 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus 3 per cent of the ether- and benzene-extracted residue of wheat embryo.

This experiment shows that hot benzene does not remove the water-soluble B from ether-extracted wheat germ directly. This is in agreement with the results obtained with beans (Chart 1, Lot 953 A, Period 2, and Chart 2, Lot 994 A, Period 2).

Lot 791 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the benzene extract of 14.28 per cent of ether-extracted wheat embryo.

Period 3.—Ration: Same as Period 2 plus 3 per cent of the ether- and benzene-extracted residue of wheat embryo.

Two rats had polyneuritis in Period 2. In Period 3 the ration caused prompt recovery. This experiment shows that benzene does not remove the water-soluble B directly from wheat embryo. The residue fed in Period 3 still contained the physiologically active substance.

Preparation of Extract and Residue Employed with Chart 6, Lots 789 A and 791 A.—1,000 gm. of wheat embryo which had been extracted 18 hours with alcohol-free ether were extracted with hot benzene continuously for 18 hours. Most of the benzene was then distilled off and the residue which was readily soluble in a little benzene was evaporated in a weighed crystallizing dish and dried *in vacuo* 24 hours. The weight of the material dissolved by benzene was 3.629 gm. This was again taken up in benzene and evaporated on dextrin, and made up to 200 gm. with dried dextrin. This preparation was employed in the ration of Lot 791 A, Period 2.

The benzene-insoluble material was used in the rations of Lot 789 A, Period 2, and also in Lot 791 A, Period 3.

Chart 7. Lots 955 A, 956 A, and 1,000 A.

Lot 955 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus the chloroform extract of ether-extracted wheat embryo equivalent to 15 per cent of wheat embryo.

Lot 956 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Digitized by Google

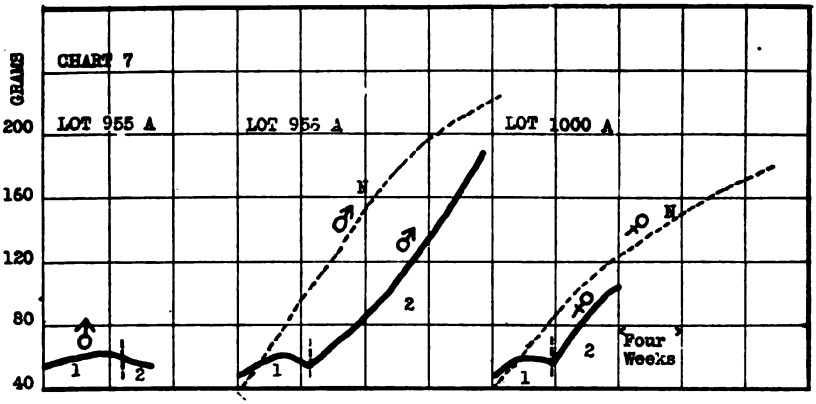
Period 2.—Ration: Same as Period 1 but with the addition of 3 per cent of wheat germ which had been extracted with ether and afterwards with hot chloroform for 18 hours.

This experiment shows that neither ether nor hot chloroform takes out the water-soluble B from wheat embryo.

Lot 1,000 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with the alcoholic extract of ether-extracted *raw* beans equivalent to 10 per cent of beans.

It has been shown previously that it is very difficult, if at all possible, to extract completely the water-soluble B from beans



by means of hot alcohol (Chart 3, Lot 951 A, Period 3). This is doubtless due to the great difficulty of grinding beans to an impalpable powder.

Preparation of the Extract and Residue Employed with Chart 7, Lots 955 A and 956 A.—589 gm. of wheat embryo previously extracted with ether for 18 hours were extracted with chloroform in three consecutive 6 hour periods. 0.838 gm. was extracted in the first two 6 hour periods and 0.218 gm. in the last. The extracts were taken up in chloroform, evaporated on dextrin, and made up to 58.9 gm. with dried dextrin. All evaporating and drying operations were carried out with the least possible exposure to heat. The extract (chloroform-soluble material) was used in the ration of Lot 955 A. The chloroform-extracted residue of wheat embryo was employed in the ration of Lot 956 A.

Digitized by Google

Preparation of the Extracts Employed with Chart 7, Lot 1,000 A and Chart 8, Lot 1,000 B.—1,052 gm. of *raw* navy beans after extraction with ether for 18 hours were extracted with hot alcohol for 18 hours in 3 consecutive 6 hour periods, changing the flasks containing the extracts at the end of each period. The first 12 hours' extraction yielded 45 gm. of extractives, the last 6 hours' 10.2 gm. The extracts were evaporated on dextrin, united, and made up to 200 gm. with dextrin. This extract was used in the ration of Chart 7, Lot 1,000 A, equivalent to 10 per cent of beans, and in Chart 8, Lot 1,000 B, equivalent to 15 per cent of beans.

Chart 8. Lots 1,000 B, 973 A, and 973 B.

Lot 1,000 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the addition of the hot alcoholic extract of 15 gm. of ether-extracted *raw* navy beans per 100 gm. of ration.

The animals in this lot which received the alcoholic extract of 15 per cent of *raw* beans were but little better off than those in Chart 7, Lot 1,000 A, which got their supply of the factor B from the alcoholic extract of 10 per cent of beans. Incomplete extraction appears to be unavoidable when beans are used.

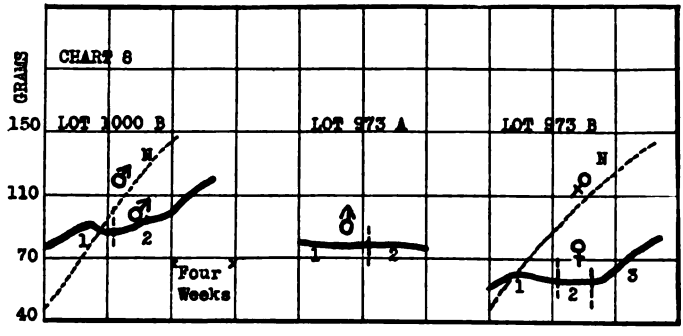
Lot 973 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the *residue* of the benzene extract of the alcoholic extract of 30 per cent of ether-extracted, *cooked* navy beans per 100 gm. of ration; *i.e.*, the alcohol-soluble, benzene-insoluble portion was fed.

At first thought this curve might give the impression that in the process of heating with water a progressive destruction of the water-soluble B takes place. 25 per cent of navy beans heated for 1½ hours at 15 pounds' pressure still induces normal growth when combined with a suitable mixture of purified foodstuffs (5) plus 5 per cent of butter fat. In Chart 1, Lot 953 A, Period 3, it was shown that the benzene extract of the alcoholic extract of 30 per cent of ether-extracted *raw* navy beans sufficed to induce good growth. The benzene extract of the alcoholic ex-

Digitized by Google

tract of 30 gm. of ether-extracted *heated* beans did not in Lot 973 B suffice to furnish enough of the factor B to induce growth, but growth at a moderate rate took place when the equivalent of 60 gm. of beans per 100 gm. of ration was included. Chart 10, Lot 933, shows, however, that even 10 per cent of *cooked* navy beans suffices to induce normal growth during 7 weeks. There may be slight destruction of the water-soluble B during cooking of beans but destruction proceeds very slowly, if at all. The explanation of the failure of the extracts of *cooked* beans to support growth as well as extracts of similar amounts of *raw* beans, lies in the failure of alcohol to extract the water-soluble B



very efficiently from *cooked* beans. This is apparently due to change in the physical properties of the bean.

Lot 973 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the benzene *extract* of the alcohol-soluble part of 30 gm. of ether-extracted *cooked* navy beans per 100 gm. of ration; *i.e.*, the alcohol-soluble, benzene-soluble portion was fed.

Period 3.—Ration: Same as in Period 2, but with the extract increased to the equivalent of 60 per cent of beans.

This experiment might seem to suggest that there is progressive destruction of the factor B on cooking the beans (compare Chart 1, Lot 953 A, Period 3). The benzene extract of the alcoholic extract of 30 gm. of *raw* beans, prepared in the same manner as the extract in this experiment, suffices for the promotion of growth. Chart 10, Lots 932 and 933, however, makes it evident

Digitized by Google

that the dietary unknown is not greatly reduced in amount by the heat treatment, for 10 per cent of *cooked* beans still supplies enough to support normal growth for 7 weeks. Rats do not eat rations which contain appreciable amounts of raw beans, so it is not possible to conduct comparable experiments with heated and unheated beans. The true explanation of these results is that apparently because of physical change in the beans during cooking alcohol fails to extract the water-soluble B after the heat treatment.

Preparation of Extracts Employed with Lots 973 A and 973 B.—918 gm. of navy beans (*cooked*) were extracted with ether for 18 hours and then with alcohol in three consecutive 6 hour periods, removing the extract at the end of each period. The first two extracts were evaporated on 100 gm. of dextrin and the alcohol was removed. There was an increase in weight of 37.1 gm. The last 6 hours' extract was evaporated on 50 gm. of dextrin giving 11.1 gm. of extract. The extracts were united and made up to 200 gm. with dextrin.

100 gm. of the above preparation equivalent to 459 gm. of beans were extracted with benzene in three consecutive 6 hour periods, removing the extract at the end of each period. The extracts were evaporated in weighed crystallizing dishes. Weight of first extract, 9.80 gm.; second extract, 0.025 gm.; third extract, trace. The extracts were taken up in benzene and evaporated on dextrin. Final weight 91.8 gm. The alcohol-soluble, benzene-insoluble portion was fed to Lot 973 A. The alcohol-soluble, benzene-soluble part was fed to Lot 973 B, first, equivalent to 30 and later raised to 60 gm. of beans per 100 gm. of ration.

Chart 9. Lots 999 A, 999 B, and 972 B.

Lot 999 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the addition of the alcoholic extract of 10 gm. of ether-extracted *cooked* navy beans per 100 gm. of ration.

Period 3.—Ration: Same as Period 2, but with the extract increased to make the equivalent of 30 gm. of *cooked* beans per 100 gm. of ration.

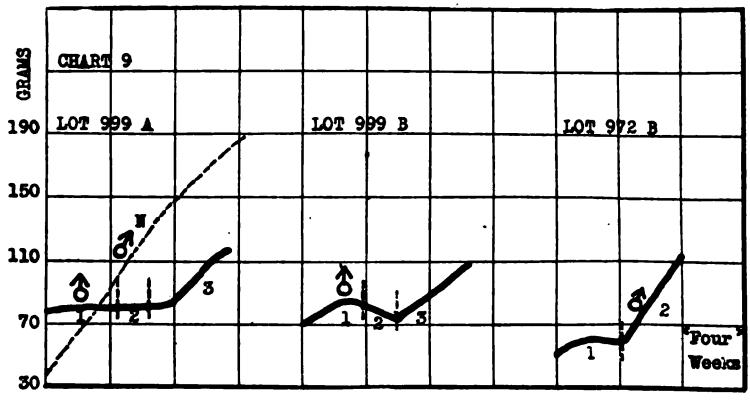
Lot 999 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Digitized by Google

Period 2.—Ration: Same as Period 1 plus the alcoholic extract of 15 gm. of ether-extracted *cooked* beans.

Period 3.—Ration: Same as Period 2, but with the extract increased to equal 40 per cent of beans in the food mixture.

This illustrates the difficulty of complete extraction of the dietary factor B from beans by alcohol after cooking. 10 per cent of beans furnish enough of this substance for normal growth during 7 weeks, but the alcoholic extraction is so incomplete when *cooked* beans are employed that the extracts used in this experiment were not adequate.



Lot 972 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the *residue* of 25 per cent of *cooked* beans, after extraction with ether and then with alcohol.

This experiment supports the evidence afforded by Chart 9, Lot 999 A and by Lot 999 B, that the failure of the alcoholic extract of *cooked* beans to induce growth is due to physical changes in the bean during cooking whereby the water-soluble B is protected from contact with the solvent and not because of destruction of the physiologically active substance.

Preparation of the Extract and Residue Employed with the Rations of Chart 9, Lots 999 A, 999 B, and 972 B.—1,176 gm. of *cooked* navy beans were extracted with alcohol-free ether for 18 hours and then with 95 per cent alcohol in three consecutive 6 hour periods, removing the flask with the extract at the end of each period to prevent excessive heating. The first 12 hours' extrac-

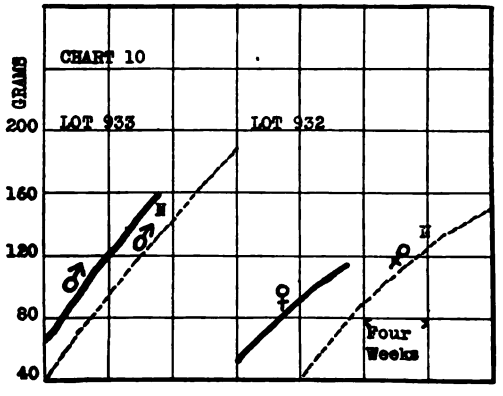
Digitized by Google

tion removed 28.4 gm. of material, the last 6 hours' 11.9 gm. The extracts were united and made up to 200 gm. with dextrin. The alcohol-soluble material was fed to Lots 999 A, Period 2, and 999 B, Period 2; the alcohol-insoluble portion of the beans was fed to Lot 972 B, Chart 9, Period 2.

Chart 10. Lots 933 and 932.

Lot 933.—Ration: Purified food mixture plus 5 per cent of butter fat, plus 10 per cent of *cooked* navy beans as the sole source of the water-soluble B.

Lot 932.—Same as Lot 933, but with 5 per cent of *cooked* beans as the source of the dietary factor B.



These curves obtained with 10 and 5 per cent respectively of *cooked* navy beans point to the great richness of the navy bean in the dietary essential water-soluble B. They indicate that probably because of a change in the physical properties of the beans during cooking, alcohol fails to extract this substance as well as it does from *raw* beans.

The stability of the dietary factor B when heated with water is a matter of great importance in practical dietetics. The stability of the factor B to heat makes possible its isolation by methods involving a considerable amount of manipulation, provided the conditions are kept suitable.

Digitized by Google

The Solubility of the Water-Soluble B as It Exists in Animal Tissues.

In our studies of the fat-soluble A we have repeatedly observed that, while ether extracts of egg yolk and the ether-soluble constituents of milk contain the unidentified A, plant fats, isolated from the seed by means of ether apparently never contain this substance (18). It remains after exhaustive extraction, in the fat-free residue of the plant tissue. We have suggested that the substance (fat-soluble A) is in chemical union in the plant tissues in a form which is not soluble in ether, and that during digestion or absorption it is set free and, being readily soluble in fats (18), thereafter accompanies the fats in the animal body. It seemed desirable to extend our observations on the solubility of the water-soluble dietary essential in organic solvents to an animal tissue, since one cannot extend with confidence the results obtained with plant tissues to tissues of animal origin, owing to the possibility that it exists in the animal cell in chemical union. Our data on this subject is still meager but that obtained with dried, unheated pig kidney will be reported here.

Chart 11. Lots 962 A, 961 A, 961 B, and 960 B.

Lot 962 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 with the addition of 6 per cent of dry, uncooked pig kidney extracted with ether and then with benzene.

This experiment is in agreement with Lot 961 A, which shows that the physiologically active substance is not extracted to an appreciable extent from animal tissue (kidney) by ether or benzene.

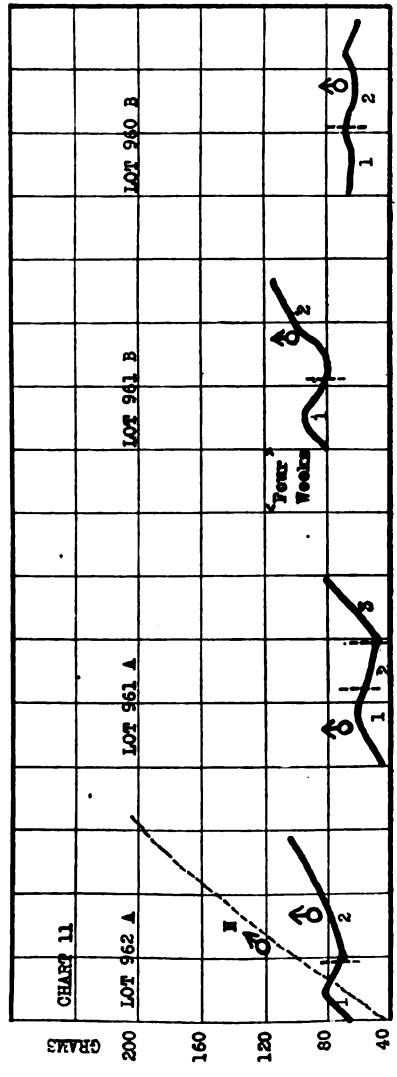
Lot 961 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the benzene extract of ether-extracted pig kidney equivalent to 6 gm. of tissue per 100 gm. of ration.

Period 3.—Ration: Same as Period 1, but with the addition of the benzene extract of the alcoholic extract of ether-extracted *raw* navy beans equivalent to 30 gm. of beans per 100 gm. of ration (the ration of Chart 1, Lot 953 A, Period 3).

The preparation from beans was introduced in Period 3 to demonstrate the capacity of the animals to grow.

Digitized by Google



Digitized by Google

Taken in connection with the results obtained in feeding the benzene extract of navy beans (Chart 1, Lot 953 A, Period 2), this experiment demonstrates the relative insolubility of the water-soluble B in hot benzene in animal tissues as well as in plant tissues.

Lot 961 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 but with 6 per cent of the residue after extraction of dry uncooked pig kidney with ether, then with acetone.

Dry, uncooked pig kidney behaves toward ether and acetone in the same way as do the navy bean and wheat germ, in that the water-soluble B is not readily extracted even by prolonged treatment with these solvents.

Lot 960 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with the addition of the acetone extract of ether-extracted pig kidney equivalent to 6 gm. per 100 gm. of ration.

This experiment shows when considered along with Lot 961 B, that acetone does not readily extract the dietary essential, water-soluble B, from dried raw pig kidney. That a small amount was extracted is indicated by the fact that the animals in this group failed to develop the loss of muscular control except in one case, and this was at the end of the 7th week after the addition of acetone extract of kidney, and at the end of the 11th week of the experiment. Animals on the ration of Period 1 never live as long as this, which shows that the acetone extract furnished a little of the water-soluble B. Lot 961 B (Chart 11) was able to make appreciable growth, and although undersized was apparently normal. After the acetone extraction, the greater part of the water-soluble B was still in the residue.

Preparation of the Extract and Residue Employed in the Ration of Lot 961 B and 960 B.—1,935 gm. of fresh pig kidney were ground up in a meat chopper and then dried at 95° for 24 hours. The weight of the dried kidney was 485 gm. which was equivalent to 25 per cent of the original weight of tissue. The dried residue was ground, then extracted with ether for 4 hours, and then ground up again and the extraction continued for an additional

Digitized by Google

14 hours. 25 per cent of ether-soluble material was thus removed, leaving 360 gm. of residue.

112.9 gm. of the above ether-free residue were extracted with acetone in three consecutive 6 hour periods, changing the flask at the end of each period. The first two 6 hour periods gave 4.572 gm. of extract, the last 6 hour period 2.26 gm. They were taken up in alcohol and evaporated on dextrin at 40°. The final weight of extracts on dextrin was 34.4 gm. This extract (acetone-soluble material) was fed in the ration of Lot 960 B, Period 2. The residue after extraction (acetone-insoluble material) was fed in the ration of Lot 961 B, Period 2.

Preparation of Extract and Residue Employed with Lots 961 A and 962 A Respectively.—122 gm. of ether-extracted kidney residue obtained as previously described were extracted with benzene in three consecutive 6 hour periods changing the flask with the extract at the end of each period. 4.10 gm. of extract were obtained in the first two 6 hour periods and 0.051 gm. in the last. Both fractions were united, and were then evaporated on dextrin yielding 28.15 gm. The benzene-soluble matter was fed to Lot 961 A, Period 2; the benzene-insoluble residue was fed to Lot 962 A, Period 2.

The Stability of the Water-Soluble B toward Nitrous Acid, Hydrochloric Acid, and Dilute Sodium Hydroxide.

The studies of Williams and his coworkers (3) and of Sullivan and Voegtlin (3) have indicated that the substance which cures polyneuritis in fowls is rapidly destroyed by even moderately dilute alkalies, but is relatively stable toward mineral acids. We have examined preparations of the water-soluble B prepared from wheat embryo by extraction with alcohol after removal of the fats by means of ether, for their power to induce growth in rats. The preparations, previous to feeding, were treated with one of the following reagents: (*a*) nitrous acid, (*b*) hydrochloric acid, (*c*) sodium hydroxide. Our results with alkalies are in agreement with those of Williams and Sullivan and Voegtlin.

Digitized by Google

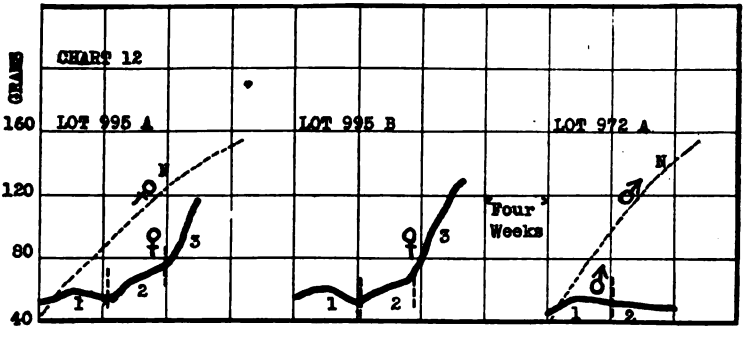
Chart 12. Lots 995 A, 995 B, and 972 A.

Lot 995 A. *Period 1.*—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 plus a preparation of water-soluble B treated 4 hours with nitrous acid. An extract equivalent to 3 per cent of wheat embryo was fed.

Period 3.—Ration: Same as Period 2, but with the water-soluble B preparation treated with nitrous acid increased to the equivalent of 10 per cent of wheat embryo.

It is possible that treatment with nitrous acid tends to destroy the dietary factor B to a slight extent, but it does so very slowly, if at all. The resistance of this substance to nitrous acid is a strong indication that it is neither a primary nor a secondary amine.



Lot 995 B. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1, but with a preparation containing the water-soluble B equivalent to 3 per cent of wheat embryo in the food mixture. This preparation was evaporated with hydrochloric acid as described below.

Period 3.—Ration: Same as Period 2 with the water-soluble B preparation increased to the equivalent to 10 per cent of wheat embryo.

It is not clear from this experiment whether or not the treatment with hydrochloric acid destroyed a part of the dietary factor B. The fact that there was no growth in Period 3 would seem to indicate that the substance is slowly inactivated. This phase of the subject will receive further study. Relatively great stability toward hydrochloric acid is clearly shown.

Digitized by Google

Treatment of Preparations Employed with Lots 995 A and 995 B.—416 gm. of wheat embryo previously extracted with ether for 18 hours, were extracted with 95 per cent alcohol for 6 hours using the Soxhlet system and for three consecutive 6 hour periods using the Caldwell system. The alcohol was distilled off from each portion separately and the extracts were brought down to dryness in a large granite pan at 40–50° in 14 hours. The residue was taken up in water and made up to 416 cc.

208 cc., equivalent to 208 gm. of wheat embryo, were transferred to an aspirating bottle and the nitrous acid gas was generated from 50 gm. of sodium nitrite, and 100 cc. of 1:1 HCl were passed in during the course of 4 hours. The gas was well washed by passing it through a strong sodium nitrite solution. The treated solution was brought down and evaporated on dextrin. The total weight of dextrin carrying the extract was brought up to 208 gm. This extract was fed in the ration of Lot 995 A (Chart 12).

The remaining 208 cc. were treated with HCl as described below and employed in the ration of Lot 995 B (Chart 12).

The solution of alcoholic extract of wheat embryo (from Preparation 995 A) was acidified with 5 cc. of C.P. HCl and evaporated on dextrin. The dry residue was made up to a final weight of 208 gm. It was rather strongly acid, since 5 gm. required 25 cc. N/14 NaOH to neutralize it to phenolphthalein.

Lot 972 A. Period 1.—Ration: Purified food mixture plus 5 per cent of butter fat.

Period 2.—Ration: Same as Period 1 with 10 per cent of wheat embryo which had been treated with 0.28 per cent sodium hydroxide at 100°C. for 1 hour.

This treatment with dilute sodium hydroxide destroyed almost completely the content of water-soluble B in the wheat embryo. This confirms the observations of others that alkalies are very destructive of this dietary essential.

Preparation of the Wheat Embryo Treated with Alkali Which Was Fed to Lot 972 A, Period 2.—550 gm. of wheat embryo, previously ether-extracted, were extracted with 95 per cent alcohol for three consecutive 6 hour periods, the flask with the extract being removed at the end of each period to minimize the possible de-

Digitized by Google

structive action of heat. The alcohol was distilled off, the extract taken up in water, and made up to 550 cc. It was distinctly acid to litmus. It was neutralized to phenolphthalein with a sodium hydroxide solution and a sufficient excess added to make it alkaline up to 0.28 per cent NaOH. At this degree of alkalinity the solution was heated for 1 hour under a reflux, then cooled, then almost neutralized with hydrochloric acid, and acidified by the addition of 12 per cent acetic acid which made it strongly acid. It was evaporated on dextrin in large granite pans and dried at 70–80° for 5 hours.

BIBLIOGRAPHY.

1. Eijkman, C., *Arch. Hyg.*, 1906, lviii, 150; *Virchows Arch. path. Anat.*, 1897, cxlviii, 523.
2. Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89. Funk, C., *Biochem. Bull.*, 1915, iv, 306. Chamberlain, W. P., Vedder, E. B., and Williams, R. R., *Philippine J. Sc., B*, 1912, vii, 40. Vedder, E. B., and Williams, R. R., *ibid.*, 1913, viii, 175, 183. Williams, R. R., *J. Biol. Chem.*, 1916, xxv, 437; xxvi, 431; 1917, xxix, 495.
3. Vedder and Williams, *Philippine J. Sc.*, 1913, viii, 175, 183. Sullivan, M. X., and Voegtlin, C., *J. Biol. Chem.*, 1916, xxiv, p. xvi.
4. McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.
5. McCollum E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix, 521.
6. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.
7. Funk, *Biochem. Bull.*, 1915, iv, 306, 311.
8. Williams, *J. Biol. Chem.*, 1917, xxix, 504.
9. Eijkman, *Arch. Schiffs. u. Tropenhyg.*, 1913, xvii, 328.
10. Funk, *J. Physiol.*, 1913, xlvi, 177.
11. Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.
12. Funk, *J. Physiol.*, 1912–13, xlv, 489.
13. Vedder and Williams, *Philippine J. Sc., B*, 1913, viii, 181. Funk, *J. Physiol.*, 1911–12, xliii, 395.
14. Benedict, S. R., *Harvey Lectures*, 1915–16, xi, 346; *J. Lab. and Clin. Med.*, 1916–17, ii, 1.
15. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 401.
16. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 181.
17. McCollum E. V., unpublished data.
18. McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333, 361.

Digitized by Google

Digitized by Google

EFFECTS OF DIET ON THE PLASMA CHLORIDES AND CHLORIDE EXCRETION IN THE DOG.

By J. HAROLD AUSTIN and LEON JONAS.*

(*From the William Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Philadelphia.*)

(Received for publication, October 17, 1917.)

In the study of the plasma chlorides in a series of nephritics Wolferth (1) recently called attention to the low figures occasionally observed in certain cases. As a rule, this finding was noted in patients who had been for some time upon a chloride-free diet. This observation we (2) have confirmed and have found the plasma chlorides as low as 4.6 gm. per liter in one case of advanced glomerulonephritis, 5 days before death. That this marked depression of the plasma chlorides might be merely the result of a long continued low chloride diet combined with a rather free administration of water seemed to us possible in spite of the well recognized tendency of the blood to maintain constant its inorganic composition. It was observed that in other cases with chronic nephritis, especially those of the so called parenchymatous type, which exhibited a tendency to elevation of the plasma chlorides, even a prolonged period of low salt diet did not bring the plasma chlorides below normal or often even to the normal figure. These, however, do not afford satisfactory evidence of the effect of a continued low chloride diet on the plasma in other diseases of the kidneys or when the kidneys are normal.

In order to obtain some data upon the point the following experiments were carried out upon dogs. The animals were placed upon one of three diets; an ordinary diet, a diet with a high sodium chloride content, or a diet poor in sodium chloride and with a free administration of distilled water. After a vari-

* Woodward Fellow in Physiological Chemistry, William Pepper Laboratory of Clinical Medicine, University of Pennsylvania.

Digitized by Google

able number of days upon the diet, a period of from 20 to 24 hours was allowed to elapse after the last feeding and the dog was then catheterized and given 100 cc. of distilled water by stomach tube to promote diuresis. About 35 minutes later blood was taken from the jugular vein for chloride analysis, and about 70 minutes after the first catheterization the animal was catheterized again and the concentration of chlorides in the urine determined.

The rate of excretion of chlorides was calculated for a 24 hour period on the basis of the excretion during the interval between the catheterizations. This portion of each experiment is designated in the protocols and table by the letter "A" following the number of the experiment. The series of "A" experiments shows the condition of the plasma chlorides and of the chloride excretion as a result of the continued use of the various diets. The main object of this part of the investigation was to determine whether following several days upon a low chloride diet the plasma chlorides would be found at a lower level than following a period on ordinary diet, and also to determine whether following a series of days on high chloride diet the plasma chlorides would be found increased, and if so whether with or without an increased rate of chloride excretion in the urine. In order to determine whether any injury to the general renal function resulted from the high salt diet, blood urea was determined in certain of the experiments and the figures were considered as evidence of the general renal functional integrity.

In order to test the immediate effect of a large dose of sodium chloride upon the plasma and upon the chloride excretion a second procedure designated "B" in the protocols and table was carried out in many of the experiments. Immediately upon completion of part "A" of the experiment 10 gm. of sodium chloride in two doses an hour apart were given by stomach tube in 5 per cent solution. Immediately after the second dose a period of urinary collection was instituted as in "A" and blood again taken for analysis in the middle of this period.

In these experiments can be noted: first, the relation between the increase in the plasma chlorides and the increase in the chloride excretion resulting from the administration of the sodium chloride; second, the effect of the previous dietary régime upon this response to the dose of chloride.

Digitized by Google

In two instances on the day following the administration of the 10 gm. of sodium chloride just described a third period of urinary collection with collection of blood was carried out to determine whether any effect of the large dose of chloride still persisted 24 hours later, either in the plasma or in the rate of chloride excretion. This part of the experiment is designated in the protocols and table as "C."

In Experiment 4, Dog I, the effect of a mild uranium nephritis in an animal on a medium chloride diet was studied for comparison with the effect of high salt feeding.

Methods.

Urine.—The chlorides were estimated by the Volhard-Harvey method (3). The concentration is indicated in the protocols and table as gm. of sodium chloride per liter of urine under "E." The rate of chloride excretion is expressed as gm. of sodium per 24 hours, calculated from the excretion in the number of minutes of urinary collection employed in each experiment; this rate of excretion is given in the protocols and table as "D."

Blood.—Blood was drawn from the jugular vein directly into a centrifuge tube containing potassium oxalate crystals beneath paraffin oil, centrifuged immediately, and the plasma pipetted from the cells (4). In the plasma so obtained chlorides were estimated by the method of McLean and Van Slyke (5). The figures are given under "N" as gm. of sodium chloride per liter of plasma.

Blood urea nitrogen was estimated on the whole blood by the method of Van Slyke and Cullen (6). The figures given are mg. of blood urea nitrogen per 100 cc. of blood. In some of the experiments blood was also drawn from the vein, allowed to clot, centrifuged, and the serum used for the estimation of electrical conductivity. Decinormal potassium chloride solution was used as a standard and determinations were made at 25°C. The specific conductivity is given under "X."

In all analyses duplicate determinations were made.

RESULTS.

The results of the fourteen experiments on the three dogs are given in the table. The detailed protocols are appended.

It will be noted that on the same ordinary régime the three dogs differed somewhat in the level of plasma chlorides; Dog I showing the highest, 6.3 gm. per liter, and Dog II the lowest, 5.9 gm. The chloride excretion in all three was small.

Digitized by Google

On a high salt diet consisting of ordinary diet plus from 5 to 10 gm. of sodium chloride daily continued from 4 to 13 days Dogs I and III showed a definite, although in the case of Dog III a very slight, increase in the level of the plasma chlorides. The study of the blood and urine was made the day following the last feeding and last dose of chloride. The urinary excretion of chloride was still definitely increased in Dog III but was normal in Dog I. Dog II developed a marked diarrhea as soon as the chloride high diet was commenced and this probably accounts for the absence of any increase in the chloride excretion and for the very slight degree of change in the plasma chlorides the day following the last dose of chloride.

In Experiment 14, 48 hours were allowed to elapse between the last dose of chloride and the study of the blood and urine, and this interval proved sufficient to allow a return of the plasma chlorides and chloride excretion to normal, in spite of the fact that the dog had received 7.5 gm. of added sodium chloride daily for 20 consecutive days.

It seems, therefore, that such increases in the plasma chlorides and chloride excretion as were observed in "A" group of experiments 24 hours after the last day of high chloride diet represent merely the persistence of the acute disturbance of these factors which follow each administration of chloride as shown in the "B" group of experiments about to be discussed and which may be seen persisting into the following day in Experiment 7, "C." There is no evidence that the continued use of a high chloride diet leads to a permanent or stable increase in the plasma chlorides or to an elevation of the chloride threshold.

The use of a low chloride diet combined with a free flushing with distilled water failed to reduce the level of the plasma chlorides below normal. In no case were we able to reduce the plasma chlorides below the original level observed in that animal. The same constancy was observed in the electrical conductivity of the serum. These experiments indicate, therefore, that in those cases of clinical nephritis, in which following the use of a low chloride diet the plasma chlorides fall distinctly below normal, this depression of the plasma chlorides is evidence of disease and not merely a consequence of the patient's régime.

In the "B" group of experiments we studied the immediate

Digitized by Google

effect of administration of 10 gm. of sodium chloride by the stomach in two doses an hour apart upon the plasma chlorides, electrical conductivity of the serum, and rate of chloride excretion. A marked rise in the plasma chlorides resulted, amounting to from 0.5 to 1 gm. per liter. This was associated with a markedly increased chloride output in the urine. Inspection of the table shows that no formula could be applied which would express the relations between the increases in these two factors either with or without inclusion of the urinary chloride concentration in the formula. In Dog I the highest chloride output, absolute and percentile, was associated with the lowest plasma chloride observed in this animal in any of the examinations immediately after chloride administration.

The electrical conductivity of the serum exhibited an increase about commensurate with that of the plasma chlorides. In Dog III, Experiment 7, when the dog had been on a diet of moderate chloride content neither the plasma nor the urinary excretion had returned to normal on the day following the administration of 10 gm. of sodium chloride (Experiment 7, "C"). In Experiment 9, however, when the dog had been on a low chloride diet, the plasma chlorides had returned to normal the day following the administration of 10 gm. of sodium chloride, although the urine still showed a somewhat increased output.

In Experiment 4, in which the effect of a mild uranium nephritis was studied, the elevation of the plasma chlorides on an ordinary diet was very marked and the chloride excretion poor. Following the administration of 10 gm. of sodium chloride by stomach, although only a moderate increase occurred in the plasma chlorides, they reached the highest figure observed in any of these experiments, but almost no response resulted in the urinary chloride excretion. The behavior of the kidney in this experiment can clearly not be interpreted as due merely to the elevation of its threshold for chlorides as the result of the nephritis. Obviously in this experiment a given increment in the level of the plasma chlorides excited much less increase in the rate of the chloride excretion than was the case in the other experiments in which the kidneys were normal.

McLean (7) has shown that in the normal animal the chloride threshold is constant and that a definite rise in plasma chlorides

Digitized by Google

provokes an increment in urinary excretion proportional to the square of the rise. The constancy of this threshold even under a prolonged régime of extremely high or extremely low chloride diet is again demonstrated in our experiments. In the pathological kidney, however, there may be either an alteration in the threshold or a disturbance in the degree of renal response to increments in the plasma chlorides above the threshold. Because of these two variables interpretation of the significance of alterations in the chloride index in pathological cases is complicated.

PROTOCOLS.

Dog I.—Female, weight 14 kilos.

Experiment 1.—Ordinary diet. On a mixed diet of table scraps. Water *ad libitum.*

Experiment 1 A.—No food or water since previous day.

Sept. 28, 11.40 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 12.15 p.m. Bled 12 cc. 12.40 p.m. Catheterized. Only 1 cc. of urine obtained. Given 100 cc. of distilled water. 1.55 p.m. Catheterized. 160 cc. of urine (135 minutes). N, 6.3; D, 0.85; E, 0.5.

Experiment 1 B.—Sept. 28. 1.55 p.m. Given 100 cc. of 3 per cent sodium chloride solution by stomach tube. 3.00 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. Catheterized. 3.25 p.m. Bled 24 cc. 4.20 p.m. Catheterized. 135 cc. of urine (80 minutes). N, 7.3; D, 34.5; E, 14.2.

Experiment 2.—High salt diet. Dog placed for 5 days (Sept. 29 to Oct. 3) on a diet of milk, 1 quart, and one dog biscuit each day. In addition given each day (except Oct. 2) 150 cc. of 5 per cent sodium chloride solution by stomach tube. Water *ad libitum.*

Experiment 2 A.—No food, water, or salt since previous day.

Oct. 4, 2.45 p.m. Catheterized. Given 90 cc. of distilled water by stomach tube. 3.18 p.m. Bled 24 cc. 3.45 p.m. Catheterized. 15 cc. of urine (60 minutes). N, 6.7; D, 0.36; E, 1.

Experiment 2 B.—Oct. 4, 3.45 p.m. Given 95 cc. of 5 per cent sodium chloride solution by stomach tube. 4.43 p.m. Same dose of sodium chloride repeated. Catheterized. 5.20 p.m. Bled 24 cc. 5.45 p.m. Catheterized. 140 cc. of urine (62 minutes). N, 7.4; D, 49.5; E, 15.2.

Experiment 3.—Low salt diet. For 10 days (Oct. 18 to Oct. 27) on a low salt diet. (Milk, 1 quart, and dried salt-free bread.) In addition given each day 150 cc. of distilled water by stomach tube.

Experiment 3 A.—No food or water since previous day.

Oct. 28, 9.45 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 10.20 a.m. Bled 16 cc. 10.50 a.m. Catheterized. 12 cc. of urine (65 minutes). N, 6.3; D, 0.1; E, 0.4.

Digitized by Google

Experiment 3 B.—Oct. 28, 10.50 a.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 11.55 a.m. Same dose of sodium chloride repeated. Catheterized. 12.30 p.m. Bled 20 cc. 1.05 p.m. Catheterized. 167 cc. of urine (70 minutes). N, 6.8; D, 53.3; E, 15.5.

Experiment 4.—Uranium nephritis. For 5 days (Oct. 29 to Nov. 3) on diet of milk, 1 quart, and one dog biscuit daily. Water *ad libitum.*

Oct. 30. Given uranium nitrate, 0.02 gm. subcutaneously.

Nov. 4. Shows a moderate cloud of albumin in urine.

Experiment 4 A.—No food or water since previous day.

Nov. 4, 12.03 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 12.37 p.m. Bled 12 cc. 1.25 p.m. Catheterized. 25 cc. of urine (82 minutes). N, 7.4; D, 0.13; E, 0.3.

Experiment 4 B.—Nov. 4, 1.25 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 2.32 p.m. Same dose of sodium chloride repeated. Catheterized. 3.10 p.m. Bled 24 cc. 3.42 p.m. Catheterized. 35 cc. of urine (70 minutes). N, 7.7; D, 0.78; E, 1.04; blood urea N, 39.

Dog II.—Female, weight 16 kilos.

Experiment 5.—Ordinary diet. Diet of milk, 1 quart, and one dog biscuit daily beginning Dec. 27. Water *ad libitum.*

Experiment 5 A.—No food or water since previous day. Dec. 29, 11.07 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 11.52 a.m. Bled 25 cc. 12.25 p.m. Catheterized. 25 cc. of urine (78 minutes). N, 5.9; D, 0.67; E, 1.46; X, 0.0129; blood urea N, 14.

Experiment 5 B.—Dec. 29, 12.25 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 1.50 p.m. Same dose of sodium chloride repeated. Catheterized. 2.32 p.m. Bled 35 cc. 3.10 p.m. Catheterized. 59 cc. of urine (80 minutes). N, 6.7; D, 17.1; E, 16.1; X, 0.0142.

Experiment 6.—High salt diet. Dog continued on the same diet of milk and dog biscuit. For 4 days (Jan. 2 to 5) received in addition sodium chloride as follows: Jan. 2 and 3, 130 cc. of 5 per cent sodium chloride solution by stomach tube; Jan. 4 and 5, 200 cc. of same. Water *ad libitum.* Diarrhea beginning Jan. 3.

Experiment 6 A.—No food, water, or salt since previous day.

Jan. 6, 11.00 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 11.35 a.m. Bled 30 cc. 12.25 p.m. Catheterized. 9.5 cc. of urine (85 minutes). N, 6.0; D, 0.56; E, 3.5; blood urea N, 10.

Dog III.—Female, weight 14 kilos.

Experiment 7.—Ordinary diet. Placed on a diet of milk, 1 quart, and one dog biscuit, beginning Dec. 27. Water *ad libitum.*

Experiment 7 A.—No food or water since previous day.

Jan. 2, 11.40 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 12.25 p.m. Bled 20 cc. 1.10 p.m. Catheterized. 5 cc. of urine (90 minutes). N, 6.0; D, 0.1; E, 1.2; X, 0.0132.

Digitized by Google

Experiment 7 B.—Jan. 2, 1.10 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 3.00 p.m. Same dose of sodium chloride repeated. Catheterized. 3.35 p.m. Bled 30 cc. 4.10 p.m. Catheterized. 32 cc. of urine (70 minutes). N, 7.0; D, 14.6; E, 22.1; X, 0.0147; blood urea N, 10.

Experiment 7 C.—Received milk, 1 quart, one dog biscuit, and water *ad libitum* at 4.30 p.m. on Jan. 2. No further food or water.

Jan. 3, 10.20 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 11.30 a.m. Bled 12 cc. 12.20 p.m. Catheterized. 76 cc. of urine (120 minutes). N, 6.3; D, 21.5; E, 23.6.

Experiment 8.—High salt diet. In addition to diet of milk and dog biscuit as above, received daily 100 cc. of 5 per cent sodium chloride solution by stomach tube. Jan. 3 to 12. Water *ad libitum.*

Experiment 8 A.—Jan. 13. No food or water. 2.18 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 2.50 p.m. Bled 30 cc. 3.22 p.m. Catheterized. 23 cc. of urine (66 minutes). N, 6.2; D, 2.8; E, 5.7; X, 0.0133.

Experiment 8 B.—Jan. 13, 3.22 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 4.05 p.m. Same dose of sodium chloride repeated. Catheterized. 4.32 p.m. Bled 20 cc. 5.20 p.m. Catheterized. 73 cc. of urine (75 minutes). N, 7.2; D, 27; E, 19.3; X, 0.0148.

Experiment 9.—Low salt diet. For 8 days (Jan. 18 to 25) given daily milk, 1 quart, and dried salt-free bread. In addition given daily 200 cc. of distilled water by stomach tube. Water *ad libitum*, but not taken.

Experiment 9 A.—No food or water since previous day.

Jan. 26, 1.32 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 2.05 p.m. Bled 30 cc. 2.35 p.m. Catheterized. 9 cc. of urine (63 minutes). N, 6.1; D, 0.4; E, 2.1; blood urea N, 10.

Experiment 9 B.—Jan. 26, 2.35 p.m. Given 100 cc. of 5 per cent sodium chloride solution by stomach tube. 3.25 p.m. Same dose of sodium chloride repeated. Catheterized. 3.55 p.m. Bled 20 cc. 4.45 p.m. Catheterized. 90 cc. of urine (80 minutes). N, 7.0; D, 25.3; E, 15.6.

Experiment 9 C.—Received milk, 1 quart, and salt-free bread at 5 p.m., Jan. 26. Water *ad libitum.* No further food or water.

Jan. 27, 9.35 a.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 10.15 a.m. Bled 20 cc. 10.45 a.m. Catheterized. 27 cc. of urine (70 minutes). N, 6.0; D, 1.2; E, 2.1.

Experiment 10.—Low salt diet. Same diet and distilled water as in Experiment 9 (Jan. 27 to Feb. 2).

Experiment 10 A.—No food or water since previous day.

Feb. 3, 2.42 p.m. Bled (No. 1) 20 cc. Catheterized. Given 100 cc. of distilled water by stomach tube. 3.30 p.m. Bled (No. 2) 30 cc. 4.07 p.m. Catheterized. 42 cc. of urine (85 minutes). No. 1: N, 6.1; X, 0.0133. No. 2: N, 6.0; D, 0.3; E, 0.4; X, 0.0132.

Experiment 11.—Low salt diet. Same diet and distilled water as in Experiments 9 and 10 (Feb. 5 to 11).

Digitized by Google

Dog.	Experiment No.	Diet.	No. of days on diet.	A. Before chloride administration.				Blood urea N.	B. After chloride administration.				C. 24 hrs. later.			
				N.	D.	E.	X.		N.	D.	E.	X.	N.	D.	E.	X.
				gm.	gm.	gm.			gm.	gm.	gm.		gm.	gm.	gm.	
I	1	Ordinary.	—	6.3	0.85	0.5	—	—	7.3	34.5	14.2	—	—	—	—	—
	2	High salt.	5	6.7	0.36	1.0	—	—	7.4	49.5	15.2	—	—	—	—	—
	3	Low "	10	6.3	0.10	0.4	—	—	6.8	53.3	15.5	—	—	—	—	—
	4	Uranium.	5	7.4	0.13	0.3	—	39	7.7	0.78	1.04	—	—	—	—	—
II	5	Ordinary.	—	5.9	0.67	1.46	0.0129	14	6.7	17.1	16.1	0.0142	—	—	—	—
	6	High salt (diarrhea).	4	6.0	0.56	3.5	—	10	—	—	—	—	—	—	—	—
III	7	Ordinary.	—	6.0	0.10	1.2	0.0132	10	7.0	14.6	22.1	0.0147	6.3	21.5	23.6	—
	8	High salt.	10	6.2	2.80	5.7	0.0133	—	7.2	27.0	19.3	0.0148	—	—	—	—
	9	Low "	8	6.1	0.40	2.1	—	10	7.0	25.3	15.6	—	6.0	1.2	2.1	—
	10	" "	7	6.0	0.30	0.4	0.0132	—	—	—	—	—	—	—	—	—
	11	" "	7	6.2	0.60	1.2	0.0133	14	—	—	—	—	—	—	—	—
	12	" "	8	6.2	0.36	0.6	0.0133	—	—	—	—	—	—	—	—	—
	13	High "	13	6.35	1.50	5.8	0.0134	14	—	—	—	—	—	—	—	—
	14	" "	6	6.2	0.50	0.7	—	13	—	—	—	—	—	—	—	—

N = gm. of sodium chloride per liter of plasma.
D = " " " " " day in urine (calculated).
E = " " " " " liter in urine.
X = Specific conductivity of serum.

Digitized by Google

Experiment 11 A.—No food or water since previous day.

Feb. 12, 3.00 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 3.45 p.m. Bled 30 cc. 4.30 p.m. Catheterized. 30 cc. of urine (90 minutes). N, 6.2; D, 0.6; E, 1.2; X, 0.0133; blood urea N, 14.

Experiment 12.—Low salt diet. Same diet and distilled water as in Experiments 9, 10, and 11 (Feb. 13 to 20).

Experiment 12 A.—No food or water since previous day.

Feb. 21. 3.12 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 3.52 p.m. Bled 25 cc. 4.37 p.m. Catheterized. 37 cc. of urine (85 minutes). N, 6.2; D, 0.36; E, 0.6; X, 0.0133.

Experiment 13.—High salt diet. Dog placed 13 days (Feb. 28 to Mar. 12) on milk, 1 quart, and one dog biscuit daily. Given 150 cc. of 5 per cent sodium chloride solution daily by stomach tube. Water *ad libitum*.

Experiment 13 A.—No food, water, or salt since previous day.

Mar. 13, 3.25 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 4.05 p.m. Bled 30 cc. 4.40 p.m. Catheterized. 13 cc. of urine (75 minutes). N, 6.35; D, 1.5; E, 5.8; X, 0.0134, blood urea N, 14.

Experiment 14.—High salt diet. Same diet and sodium chloride as in Experiment 13 (Mar. 14 to 19). Same diet, no sodium chloride, Mar. 20.

Experiment 14 A.—No food or water since previous day.

Mar. 21, 2.00 p.m. Catheterized. Given 100 cc. of distilled water by stomach tube. 2.35 p.m. Bled 30 cc. 3.05 p.m. Catheterized. 32 cc. of urine (65 minutes). N, 6.2; D, 0.5; E, 0.7; blood urea N, 13.

CONCLUSIONS.

1. The level of plasma sodium chloride 24 hours after feeding varied in three dogs from 5.9 to 6.3 gm. per liter, but was quite constant for any one animal.

2. By administering large amounts of sodium chloride in solution by stomach tube, the level of the plasma chlorides can be raised in the dog within 1½ hours, 1 gm. per liter. The return to the previous level occurs within from 24 to 48 hours.

3. It is not possible to raise the level of the plasma chlorides other than in this transient manner by a prolonged régime on a high chloride diet, or to lower the plasma chlorides below the normal level even by a prolonged régime on a very low chloride diet.

4. In certain nephritic animals with impaired chloride excretion, whatever may be the hypothetical chloride threshold, a given increment in the plasma chloride concentration excites less increment in the rate of chloride excretion than it would in a normal animal.

Digitized by Google

BIBLIOGRAPHY.

1. Wolferth, C. C., A clinical study of chloride excretory function, *Am. J. Med. Sc.*, 1917, cliv, 84.
2. Stengel, A., Austin, J. H., and Jonas, L., A comparison of the functional and anatomical findings in a series of cases of renal disease, *Arch. Int. Med.*, 1918, in press.
3. Harvey, S. C., The quantitative determination of the chlorides in the urine, *Arch. Int. Med.*, 1910, vi, 12.
4. Austin and Jonas, Clinical studies of acidosis, *Am. J. Med. Sc.*, 1917, cliii, 81.
5. McLean, F. C., and Van Slyke, D. D., A method for the determination of chlorides in small amounts of body fluids, *J. Biol. Chem.*, 1915, xxi, 361.
6. Van Slyke, D. D., and Cullen, G. E., A permanent preparation of urease, and its use in the determination of urea, *J. Biol. Chem.*, 1914, xix, 211.
7. McLean, F. C., The numerical laws governing the rate of excretion of urea and chlorides in man, *J. Exp. Med.*, 1915, xxii, 212.

Digitized by Google

Digitized by Google

STUDIES OF ACID PRODUCTION.

II. THE MINERAL LOSS DURING ACIDOSIS.*

By MARGARET SAWYER, L. BAUMANN, and F. STEVENS.

(From the Departments of Home Economics and Internal Medicine, State University of Iowa, Iowa City.)

(Received for publication, November 19, 1917.)

The effect of acid administration and increased acid production on the excretion of certain minerals and nitrogen has been repeatedly studied. Recent discussions of the literature and bibliographies will be found in the publications of Forbes and Keith,[1] Givens and Mendel,[2] and Stehle.[3]

A survey of the literature failed to show a record of a complete experiment on the normal growing human organism. As children are peculiarly prone to acidosis it appeared desirable to supply the missing data. It was with this object in view that the following experiments were undertaken.

Methods.

The observations were made in a metabolism ward on two male children aged 5 and 8 years, and weighing 22.7 and 23 kilos, respectively. The boys were under the supervision of a special nurse. The food was prepared by a trained dietitian. Each period of observation lasted 3 days.

Each child consumed 436 gm. of egg white, 74 gm. of egg yolk, 414 gm. of milk, 64 gm. of pure sucrose, 60 gm. of pure lactose, 2 gm. of pure sodium chloride, and 3 gm. of agar, daily during the normal period. This amount of food contained 324 protein, 385 fat, 593 carbohydrate calories, and 12.6 gm. of nitrogen. The energy intake was equivalent to 56 calories per kilo. During the period of high fat feeding the protein calories remained the same, but the carbohydrate calories were reduced to 85,

* The expenses of the research were met in part by a grant from the research fund of the Graduate College of the State University of Iowa.

[1] Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station Technical Bull. 5*, 1914.

[2] Givens, M. H., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 421.

[3] Stehle, R. L., *J. Biol. Chem.*, 1917, xxxi, 461.

Digitized by Google

while the fat calories were increased to 857. This was accomplished, by replacing the lactose and sucrose of the normal period, with an isodynamic quantity of pure butter fat (51 gm.) prepared according to the directions of Osborne and Mendel.[4] The mineral, calorie, and nitrogen intake, therefore, was practically identical during both periods.

The nitrogen and mineral content of the food was determined by analysis. The figures were compared with those obtained by calculation from the tables of Rose[5] (see Table I).

The urine of each voiding was separately collected in a Mason jar and preserved in the refrigerator after the addition of toluene. The 24 hour volume was diluted to 1 liter and 100 cc. were removed for the determination of titratable acidity, ammonia, creatine, creatinine, and acetone. The remaining urine of the 3 days (2,700 cc.) was united and analyzed for nitrogen and minerals. The feces were separated with the aid of carmine. The latter was administered, with the agar and milk, without other food, thus securing a sharp separation of the periods. The feces of a period were triturated with distilled water until a homogeneous suspension was obtained. Weighed aliquot portions were analyzed. The determinations of the minerals in the food, urine, and feces were carried out as described in a former article.[6] The sulfur of the food and feces was conveniently determined by the Benedict method, after treating the material with fuming nitric acid in a Kjeldahl flask. The modified oxidizing solution, recommended by Denis,[7] was found to be preferable to the original.

A record of the analytical data and results will be found in the following tables.

TABLE I.

*The Nitrogen and Mineral Content of the Daily Food.**

	Nitrogen.	Sulfur.	Phosphorus.	Chlorine.	Calcium.	Magnesium.	Sodium.	Potassium.
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Calculated, Rose[5]...	12.6	1.11	0.7688	2.44	0.6496	0.0958	1.73	1.357
Found.								
Normal period....	12.06	1.13	0.8799	2.31	0.7024	0.1465	1.781	1.261
High fat "	11.93	1.11	0.8631	2.49	0.6430	0.1046	1.762	1.207

* The addition of agar to the food probably accounts for the disagreement between the calculated and found values for calcium, magnesium, and phosphorus.

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913–14, xvi, 423.

[5] Rose, M. S., A Laboratory Handbook for Dietetics, New York, 1912, 109.

[6] Baumann, L., and Howard, C. P., *Arch. Int. Med.*, 1912, ix, 665.

[7] Denis, W., *J. Biol. Chem.*, 1910–11, viii, 401.

Digitized by Google

TABLE II.

Record of the Excretion of Certain Blood and Urine Constituents during Periods of Normal and High Fat Feeding.

Day.	Diet.	Titratable acid.*	Ammonia.*	Creatinine.	Creatine.	Acetone (qualitative).	Carbonate of plasma.	Remarks.
		cc.	cc.	mg.	mg.		vol. per cent	
1	Normal.	125	385	276	192			Subject C. G., aged 5 yrs.;
2	"	135	385	274	194			weight 22.7 kg. Physi-
3	"	120	395	274	174		50	cal examination, nega-
								tive.
4	High fat.	140	330	272	178	+		During the high fat pe-
5	" "	170	500	256	236	++		riod, restlessness and a
6	" "	240	815	244	290	+++	29	slight increase of the
7	Normal.		818	284	202			pulse rate were ob-
								served.
1	Normal.	100	465	332	96			Subject R. W., aged 8
2	"	110	455	362	86			yrs.; weight 23.2 kg.
3	"	100	465	336	143		49	Physical examination
4	High fat.	120	390	362	124	+		showed scars of mas-
5	" "	120	425	342	216	++		toid and tonsillectomy
6	" "	145	530	334	250	+++	37	operations.
7	Normal.		630	361	297			

* The titratable acid and ammonia are expressed in terms of 0.1 N solution.

Digitized by Google

TABLE III.

The Intake, Output, and Balance of Minerals and Nitrogen during 3 Day Periods of Normal and High Fat Feeding.

		Nitrogen.	Sulfur.	Chlorine.	Phosphorus.	Sodium.	Potassium.	Calcium.	Magnesium.
C. G. Normal diet.	Food.....	36.18	3.399	6.930	2.6397	5.343	3.783	2.1072	0.4359
	Urine.....	34.17	3.27	7.242	2.019	5.100	3.528	0.8334	0.210
	Feces.....	0.156	0.1671	0.099	0.6671	0.0873	0.561	1.203	0.1503
	Balance.....	+1.845	−0.0383	−0.411	−0.0537	+0.1657	−0.306	+0.0708	+0.0792
C. G. High fat diet.	Food.....	35.79	3.33	7.458	2.5893	5.286	3.621	1.929	0.3138
	Urine.....	38.64	3.51	7.029	2.6055	5.724	4.260	1.3158	0.300
	Feces.....	2.145	0.2931	0.066	0.99	0.0609	0.7983	1.815	0.2799
	Balance.....	−4.975	−0.473	+0.363	−1.0057	−0.4989	−1.4373	−1.2018	−0.2661
R. W. Normal diet.	Food.....	36.18	3.399	6.93	2.6397	5.343	3.783	2.1072	0.4359
	Urine.....	35.76	3.345	7.029	1.965	5.082	3.147	0.4461	0.2424
	Feces.....	1.4565	0.216	0.0182	1.0191	0.0288	0.6411	1.7100	0.1452
	Balance.....	−1.03	−0.162	−0.1172	−0.3423	+0.2322	−0.0051	−0.0488	+0.0483
R. W. High fat diet.....	Food.....	35.79	3.33	7.458	2.5893	5.286	3.621	1.929	0.3138
	Urine.....	41.28	3.693	7.794	2.472	6.396	3.711	0.6966	0.3018
	Feces.....	1.107	0.1182	0.0156	0.8979	0.0369	0.9189	1.1230	0.3939
	Balance.....	−6.597	−0.481	−0.3512	−0.7806	−1.1460	−1.0889	+0.1084	−0.3819

Digitized by Google

TABLE IV.

The Mineral Excretion Expressed in Cc. 0.1 N Solution.

		Sulfur.	Phosphorus.†	Chlorine.	Calcium.	Magnesium.	Sodium.	Potassium.	Ammonia.
C. G. Normal diet.	Urine.*	1,853	1,302	2,042	416	203	2,217	902	1,165
	Feces..	104	430	28	600	124	38	143	
	Total..	1,957	1,732	2,070	1,016	327	2,255	1,045	
	Total acid.... 5,759								
	" base.... 5,808								
C. G. High fat diet.	Urine..	2,072	1,681	1,982	656	247	2,489	1,090	1,645
	Feces..	183	644	19	905	230	27	204	
	Total..	2,255	2,325	2,001	1,561	477	2,516	1,294	
	Total acid.... 6,581								
	" base.... 7,493								
R. W. Normal diet.	Urine..	1,983	1,268	1,982	223	199	2,209	805	1,385
	Feces..	138	657	5	853	119	13	164	
	Total..	2,121	1,925	1,987	1,076	318	2,222	969	
	Total acid.... 6,033								
	" base.... 5,970								
R. W. High fat diet.	Urine..	2,233	1,593	2,198	348	248	2,781	949	1,345
	Feces..	74	579	4	560	324	16	235	
	Total..	2,307	2,172	2,202	908	572	2,797	1,184	
	Total acid.... 6,681								
	" base.... 6,806								

* The acid equivalent of the total sulfates only is recorded.

† Phosphoric acid was calculated as a dibasic acid.

DISCUSSION.

Inspection of the tables shows that C. G., the younger child, responded promptly to the high fat diet with a marked increase in nitrogen, minerals, ammonia, titratable acid, and creatine.

During the preliminary normal period he excreted the equivalent of 5,759 cc. of mineral acid and 5,808 cc. of base; that is, 49 cc. of base served for the neutralization of organic acid. The titratable acidity, 380 cc., plus 49 cc. indicates the approximate organic acid excretion (429 cc.). During the period of high fat

Digitized by Google

feeding, 6,581 cc. of mineral acid and 7,493 cc. of base were excreted; that is, an excess of 912 cc. of base for the neutralization of organic acid. The sum of the titratable acid (550 cc.) and the excess of base excreted (912 cc.) is 1,462 cc., which is the amount of organic acid excreted during the 3 days of this period.

R. W. was less susceptible to the change in diet. The excretion of nitrogen, minerals (except calcium), creatine, and titratable acid was increased, but not to the same extent as in the former child. During the normal period 6,033 cc. of acid and 5,970 cc. of base were excreted; that is, 63 cc. of acid in excess of base. The titratable acid of the urine (310 cc.) minus the excess of mineral acid (63 cc.) leaves 247 cc. for the organic acid elimination. During the high fat period 6,681 cc. of acid and 6,806 cc. of base were excreted. The titratable acid of the urine (385 cc.) plus the excess of base over mineral acid excretion (125 cc.) makes 510 cc., which is the organic acid excretion for this period. The values of the plasma carbonates for the high fat periods were in keeping with the urinary findings. Under similar conditions, C. G., the more responsive case, showed a lowering of the carbonate value amounting to 21 volume per cent, whereas R. W.'s plasma carbonates decreased by 12 volume per cent.[8]

The increased elimination of nitrogen during the periods of high fat feeding may possibly be ascribed to an accelerated rate of conversion of protein into carbohydrate. The ammonia in the urine of the more responsive case accounts for but 10 per cent of the excess nitrogen eliminated. The ratio of sulfur to nitrogen lost is about 1:17 and 1:21 respectively.

If we assumed that the lost nitrogen was derived from muscle exclusively, then in the case of C. G. about 320 gm. of muscle tissue would have been catabolized. The minerals contained therein would be entirely inadequate to account for the calcium, magnesium, and phosphorus losses. One naturally associates this trio of elements with bone and it is not unlikely that they were derived from this source. The excess of calcium which was excreted during the period of high fat feeding served

[8] The variation in the response of children to acid-producing diets is marked. We have recorded observations concerning this point on a larger series of children in a forthcoming number of the *Am. J. Dis. Child.*

Digitized by Google

to neutralize more acid (545 cc.) than the surplus of ammonia (480 cc.).

Fitz, Alsberg, and Henderson[9] and others have pointed out the increased elimination of acid phosphates during periods of acidosis. This may account for part of the increased excretion of sodium, potassium, and phosphorus during the period of high fat feeding.

The phosphorus loss of the older child, with the more moderate acid production, was less than one-half that of the other. Calcium was actually retained. The loss of sodium and chlorine, however, was more marked. The diminished excretion of sodium chloride, by the younger child, with the more marked acid production, may possibly be explained by the observation of Fischer[10] that acidosis is associated with a decreased sodium chloride elimination.

The above observations, largely confirmatory in nature, indicate that a considerable loss of minerals, especially *phosphorus* and *calcium*, may accompany short periods of increased acid production in children. The excretion of these elements, in the two cases studied, varies directly with the severity of the acidosis. The pronounced loss of nitrogen during the high fat period is a field for further investigation.

In conclusion, we wish to thank Drs. Dean, Steindler, and Beifeld for their cooperation, and Miss Helen Mougey for the care exercised in the preparation of the diets.

[9] Fitz, R., Alsberg, C. L., and Henderson, L. J., *Am. J. Physiol.*, 1907, xviii, 113.

[10] Fischer, M. H., *J. Am. Med. Assn.*, 1915, lxiv, 325.

Digitized by Google

Digitized by Google

LECITHIN. I.

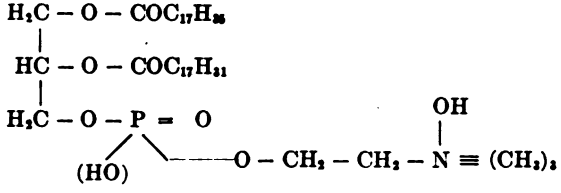
"HYDROLECITHIN" AND ITS BEARING ON THE CONSTITUTION OF CEPHALIN.

By P. A. LEVENE and C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, December 1, 1917.)

The recent investigations on the chemical structure of lecithin have resulted in many important contributions, all of which point to the correctness of the generally accepted view of its molecular structure,



However, a scrutiny of all the work on lecithin reveals a remarkable incompleteness of each individual investigation. A rigorous proof of the accepted theory requires for lecithin an elementary composition of C = 65.60, H = 10.79, N = 1.74, P = 3.86. It further requires that the nitrogen of the molecule should be composed entirely of choline. Hence, lecithin should not contain even a part of its nitrogen in the form of free amino groups. Still further, it requires a proof of the identity of the fatty acids with those accepted by theory, and finally it requires the isolation of the glycerophosphoric acid.

The work up to the present has satisfied many of the requirements. The fatty acids and the glycerophosphoric acid have been identified; and MacLean has prepared, at least once, a sample of lecithin that was free of amino nitrogen. However, this one sample has been incompletely analyzed. All other samples of lecithin prepared by various workers contained amino

Digitized by Google

nitrogen in their molecule, and from the standpoint of elementary analysis showed a marked disagreement with the theory. This is well illustrated by the following table.

Author.	Source.	C	H	N	P	Choline.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Thudichum[1].....	Brain.	66.75	18.67	1.81	4.00	—
Baskoff[2].....	Liver.	64.64	10.71	1.95	4.00	—
Heffter[3].....	"	—	—	—	—	25
Stern and Thierfelder[4].....	Egg.	64.63	10.96	1.79	3.95	—
MacLean[5].....	"	64.18	10.60	1.87	3.95	66
Erlandsen[6].....	Heart.	66.29	10.17	1.87	3.95	42
MacLean[7].....	"	66.27	10.32	1.85	3.97	41.4
Eppler[8].....	"	66.46	10.69	1.87	4.03	—
MacLean[7].....	"	—	—	1.89	4.04	68
"	Not given.	—	—	1.85	4.00	66
"	From $CdCl_2$ salt.	—	—	1.87	4.15	98.7

The analytical data obtained by Ritter[9] on hydrolecithin showed better agreement with the theory, and one might be inclined to regard the material of Ritter as such that it contained all the necessary proof in favor of the conventional theory. Unfortunately, Ritter did not determine the amino nitrogen content of the reduced lecithin, and hence failed to furnish definite proof of its purity.

Indeed, the present report contains data unmistakably proving that hydrolecithin of an elementary composition fully harmonizing with the theory may be and generally is impure, containing between 10 and 20 per cent of its nitrogen in the form

[1] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884.

[2] Baskoff, A., *Z. physiol. Chem.*, 1907, lvii, 395.

[3] Heffter, A., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 100.

[4] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 381.

[5] MacLean, H., *Z. physiol. Chem.*, 1908, lv, 360; 1909, lix, 223; *Biochem. J.*, 1909, iv, 38, 240.

[6] Erlandsen, A., *Z. physiol. Chem.*, 1907, li, 71.

[7] MacLean, *Biochem. J.*, 1915, ix, 364.

[8] Eppler, J., *Z. physiol. Chem.*, 1913, lxxxvii, 241.

[9] Ritter, F., *Ber. chem. Ges.*, 1914, xlvii, 530. Cf. *Reidel's Ber.*, 1913, lvii, 20; 1914, lviii, 15.

Digitized by Google

of amino nitrogen. Thus the task of the preparation of lecithin having a composition required by the theory and at the same time free of impurities has not yet been accomplished. Efforts in this direction are now in progress in this laboratory.

However, the present finding has a great significance because of its bearing on the structure of cephalin, and the work is presented in its present incomplete state because of this significance. The remarks made earlier in this communication regarding lecithin apply also to cephalin. On the basis of recent work on the hydrolytic products of the substance, a certain structural formula has been assumed. This formula requires an elementary composition of C = 66.17, H = 10.57, N = 1.88, and P = 4.17. However, all samples analyzed, beginning with Thudichum and up to the present by the most recent investigators,[10] consistently had the average composition of C = 60.00, H = 9.30, N = 1.80, and P = 3.80.

On the basis of these considerations, one may argue that if cephalin and lecithin both had the composition required for them by the theory, then a mixture of the two should possess practically the same elementary composition as either one of them in the pure state. On the other hand, if lecithin possessed the composition assumed by the theory and cephalin that found empirically, then a mixture containing 80 per cent of the former and 20 per cent of the latter should possess a carbon content of 64.56 per cent instead of 65.35 per cent. Conversely, if a mixture of the two reduced substances possessed an elementary analysis of C = 65.30, H = 11.20, N = 1.75, P = 3.85, it would justify the conclusion that both lecithin and cephalin possess the composition assumed for them by the theory.

The material analyzed by us contained 80 per cent of lecithin and 20 per cent of an impurity. It was found that the material yielded on hydrolysis besides the choline also the base aminoethanol. Hence it was reasonable to assume that the 20 per cent of impurity consisted of cephalin. If cephalin had the composition found by experience then a substance consisting of 80 per cent of hydrolecithin and 20 per cent of cephalin should have an elementary composition of C = 64.56, H = 10.49, N = 1.75, P =

[10] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 41.

Digitized by Google

3.84. On the other hand, if both lecithin and cephalin possess the structure assigned to them by theory then the above mixture should have the elementary composition found by experiment. Thus the facts presented in this report furnish evidence in favor of the prevailing theory of the molecular structure of lecithin and of cephalin; they also indicate the method by which the pure reduced cephalin may eventually be obtained. Efforts in this direction are now in progress.

In this connection it may be recalled that the product obtained on reduction of cephalin with hydrogen gas in the presence of palladium contained from 62 to 63 per cent of carbon in its molecule, thus approaching nearer than the non-reduced cephalin the theoretical value. The non-reduced cephalin generally obtained is undoubtedly an altered and perhaps oxidized form of the original substance. The nature of the alteration is not known as yet.

EXPERIMENTAL.

Hydrolecithin was first prepared by Paal and Oehme;[11] they reduced an alcoholic solution of egg lecithin with hydrogen and colloidal palladium. The product was obtained as microscopic compact crystals, which sintered at 83–84°, and decomposed over 150° with blackening. Upon hydrolysis the hydrolecithin gave a mixture of stearic, palmitic, and probably myristic acids. The following year Ritter[9] reduced lecithin which had been prepared from fresh, dry egg yolk as follows: The egg yolk was first extracted with petroleum ether and then with ether; the ethereal extract was concentrated and the residue was then extracted with methyl alcohol. The hydrolecithin prepared in this way proved to be distearylhydrolecithin; that is, on hydrolysis it yielded only stearic acid.

Hydrolecithin has been prepared in our laboratory, by Paal's method, from various fractions of egg lecithin, and from lecithin of brain and other organs. During the course of the work it has been noted that lecithin, which has been washed according to MacLean's[7] method, in which the lecithin is ground up with a little water and precipitated with acetone, is reduced more rapidly and more completely than unwashed lecithin. Also, it was

[11] Paal, C., and Oehme, H., *Ber. chem. Ges.*, 1913, xlvi, 1297.

Digitized by Google

found that the addition of 1 to 2 per cent of acetic acid to the alcoholic solution facilitated the reduction. With fairly concentrated solutions the hydrolecithin separates out during the course of the reduction. This product was brought into solution by warming, filtered from the coagulated palladium, cooled to 0°, and the material which separated out crystallized from dry methyl ethyl ketone until the composition was constant. In most cases it was easily possible to obtain material with correct analytical figures for carbon and hydrogen. In some cases, however, this was impossible, even after repeated crystallization.

Hydrolecithin crystallizes well from methyl ethyl ketone, in which it is insoluble in the cold; it softens between 80 and 90°, turns brown about 100°, starts to melt about 200°, and runs down the tube, giving a dark red liquid, at 235°. The figures vary somewhat, depending upon the rate of heating. The optical activity was determined in chloroform solution and varied between + 5.2° and + 5.4°. The presence of the amino nitrogen-containing body did not seem to affect the value for $[\alpha]_D^{20}$.

$$\text{Sample } 400\ [\alpha]_D^{20} = \frac{9.7822 \times 0.18°}{0.5 \times 0.3254} = +5.4°$$

$$\text{“ } 399\ [\alpha]_D^{20} = \frac{9.2784 \times 0.21°}{0.5 \times 0.3734} = +5.22°$$

$$\text{“ } 429\ [\alpha]_D^{20} = \frac{9.2150 \times 0.24°}{0.5 \times 0.4170} = +5.3°$$

$$\text{“ } 457\ [\alpha]_D^{2} = \frac{9.330 \times 0.23°}{0.5 \times 0.4120} = +5.20°$$

Analysis of these samples gave the following values:

Sample.	C	H	N	P	Ash.	NH_2—N
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
400	65.20	10.89	2.00	4.00	10.61	
399	65.59	11.16	2.37	3.85	10.38	
429	65.60	11.03	1.96	3.79	9.75	7.00
508	65.50	11.30	1.80	3.90	—	20.00
492	—	—	1.98	3.98	9.66	6.00
Theory.	65.37	11.23	1.74	3.84		0

Digitized by Google

Hydrolysis of Hydrolecithin.

A large quantity (200 gm.) of hydrolecithin was prepared for the purpose of studying its hydrolytic products. This analyzed as follows:

Sample..	C	H	N	P	NH_3–N
508	65.50	11.30	1.80	3.90	20.00

100 gm. of this material were hydrolyzed by boiling with 1 liter of 3 per cent sulfuric acid for 8 hours.[7]

The fatty acid fraction was filtered off and recrystallized from acetone, after boiling about 2 hours with animal charcoal. Again recrystallized from acetone, the acid melted at 69–70°, and on combustion and titration gave figures for *stearic acid*. This confirms Ritter's observation that it is possible to obtain pure distearylhydrolecithin.

The aqueous filtrate was freed from sulfuric acid by the addition of barium hydroxide, concentrated *in vacuo*, precipitated with basic lead acetate, the filtrate freed from lead, and used for the determination of aminoethyl alcohol according to Thierfelder and Schulze.[12] The ethereal extract was concentrated, taken up in water, and the amino nitrogen determined. The theoretical amount of gold chloride was added to the acidified solution. The gold salt separated as long needles after standing 2 days in a desiccator over sulfuric acid. It melts at 184–186°. Trier[13] gives 186–187° for the gold chloride salt of aminoethyl alcohol

[12] Thierfelder, H., and Schulze, O., *Z. physiol. Chem.*, 1916, xcvi, 296.

This method depends upon the fact that calcium oxide does not liberate choline from its hydrochloride, but does free aminoethyl alcohol. The concentrated solution of the mixed hydrochlorides is rubbed up with pure calcium oxide until it is a dry powder and extracted with ether in a Soxhlet apparatus; the flask should contain 0.1 N sulfuric acid to bind the base, otherwise considerable loss occurs. After 27 hours about 96 per cent of the alcohol has been extracted by the ether. The choline may then be extracted with hot alcohol.

[13] Trier, G., *Z. physiol. Chem.*, 1911, lxxiii, 383; 1911–12, lxxvi, 496.

Digitized by Google

hydrochloride, and Knorr[14] gives about 190° for the synthetic product. It was analyzed by heating to constant weight.

0.2008 gm. substance gave 0.0990 gm. Au.
0.2075 " " " 0.1016 " "

	Calculated:	Found:	
Au.....	49.17	49.35	48.96

[14] Knorr, L., *Ber. chem. Ges.*, 1897, xxx, 913.

Digitized by Google

Digitized by Google

THE COLORIMETRIC DETERMINATION OF HEMOGLOBIN.*

By WALTER W. PALMER.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, November 30, 1917.)

The method to be described for the determination of hemoglobin depends upon the comparison, in a colorimeter, of carbon monoxide hemoglobin solutions, one of which has a known hemoglobin content. Hoppe-Seyler (1) was the first to describe carbon monoxide hemoglobin and to make use of this stable combination for estimating the hemoglobin content of blood. He devised a "double pipette" for comparing the unknown carbon monoxide hemoglobin solution with the standard carbon monoxide hemoglobin solution, prepared from hemoglobin crystals; but the method never came into general use, because of the many technical difficulties involved. Haldane (2) suggested a much simpler method for comparing carbon monoxide solutions, using the apparatus employed by Gowers (3) for comparing oxyhemoglobin solutions with a picro-carmine standard. This apparatus was later employed by Sahli (4) who prepared an acid hematin standard by adding dilute hydrochloric acid to blood.

A critical discussion of the various methods in use for the estimating of hemoglobin is beyond the scope of this paper. As Haldane (2) has pointed out, artificially colored solutions and tinted glass present great difficulties in standardization with a definite strength of hemoglobin solution. With a certain strength of color solution or tinted glass, it is possible to imitate quite perfectly the tint of a given hemoglobin solution provided the quality of light remains the same. Any variation from these standard conditions, either in quality of light or strength of hemoglobin in solution leads to serious errors. Haldane at-

* A brief report of this paper is given in *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 175.

Digitized by Google

tempted to overcome the objection by preparing a carbon monoxide hemoglobin standard (1 per cent solution of a blood having an oxygen capacity of 18.5 per cent) which he considered permanent when kept sealed in a small test-tube in an atmosphere of carbon monoxide. 0.020 cc. of blood, placed in a similar sized and shaped graduated (on scale of 100) test-tube with a small amount of water saturated with carbon monoxide by means of ordinary illuminating gas, are diluted with water, drop by drop, until the unknown and standard tubes match in color, whence the percentage of hemoglobin in the unknown may be read off on the graduated scale. In principle, Haldane's method is sound; but certain practical difficulties arise. The standard is not so permanent as was at first thought, and, when water is used, it has been shown by Krogh (5) that the full color of the solution is slow in reaching its maximum. The further criticism is that the method is time-consuming and cumbersome—adding water, drop by drop, and shaking after each addition. Also the color comparison in the two tubes is not sharp. This method has one distinct advantage in that it may be used for the determination of hemoglobin in any species of animal.

Sahli employed Haldane's apparatus and technique; except that he used, as a standard, blood to which dilute hydrochloric acid had been added. There are three serious objections to Sahli's method: first, the standard is not permanent; second, there is considerable delay in the development of the maximum or permanent color, amounting, according to Meyer and Butterfield (6), in some instances to 20 per cent; and third, it cannot be used for the blood of different species.

The spectrophotometer has undoubted accuracy in the hands of skilled operators; but the expense and unavailability together with the difficult technique involved, make it impracticable for general use.

The great variety of methods and apparatus which have been proposed offer eloquent testimony to the unsatisfactory means for the determination of hemoglobin. There is great need for a rapid, accurate, and universally standard method for the estimation of hemoglobin in experimental work and the study of blood diseases in the clinics.

The method which we have found to fulfil the above conditions is as follows:

Digitized by Google

Method.

Blood is obtained in the usual manner by pricking the finger or lobe of the ear. A 1 per cent solution of blood is made by drawing 0.05 cc. into a special pipette and transferring into 5 cc. of 0.4 per cent ammonia solution—accurately measured with a calibrated pipette or burette into a 12 x 120 mm. test-tube. The blood pipette is rinsed out by drawing into it two or three times the ammonia solution. Ordinary illuminating gas is bubbled rapidly through the ammonia blood solution for 30 seconds, after which, it is compared in a Duboscq colorimeter with a standard carbon monoxide hemoglobin solution set at 10. The average of at least four readings is taken. The calculation is simple, $\frac{10}{R} \times 100 =$ per cent hemoglobin.

Manner of Obtaining Blood.—With sufficient care the usual clinical method for obtaining small amounts of blood by pricking the ear or finger is satisfactory. A free flow is essential. Any undue manipulation or squeezing of the part should be avoided because an error of 5 or 10 per cent may be introduced by diluting the blood with tissue juice. Where there is marked anemia requiring larger amounts of blood than 0.05 cc., or where there is difficulty in obtaining blood from the ear or finger, venous puncture should be used, coagulation being prevented with oxalate or citrate salts. It is often practical and convenient to combine the determinations of hemoglobin with other blood analyses, where venous puncture is required. If blood has been drawn by venous puncture care must be taken that the corpuscles and serum are well mixed before filling the pipette. The blood should never be shaken violently before measuring, because it becomes filled with air bubbles. The mixture of corpuscles and serum may best be accomplished by first giving the receptacle a circular motion and finally stirring briskly with a glass rod or measuring pipette which is filled while stirring the blood.

Pipette for Measuring Blood.—The pipettes are made of millimeter glass tubing calibrated to contain 0.05 cc. and 0.10 cc. The pipettes are easily made in any laboratory from straight tubing, and require no blowing, the point being rounded off on an emery wheel. In this way time and expense are saved, since pipettes

Digitized by Google

obtained from glass blowers require recalibration before use. It has been found that water may be used for this calibration, as pipettes which have been calibrated with both mercury and water check sufficiently well. The advantage of having the pipette calibrated to contain 0.10 cc. as well as 0.05 cc. is obvious. In bloods with a low hemoglobin content, 0.05 cc. may not be sufficient to give the color necessary for accurate color comparison in the colorimeter. A pipette of this type, and used in the manner described, is capable of measuring 0.05 cc. of blood with an accuracy of 0.2 per cent.

Ammonia Solution.—Ammonia solutions, containing 4 cc. of strong ammonia in 1 liter of water, suggested by Krogh (5) are used, because the full color of the carbon monoxide hemoglobin develops at once.

Saturation with Carbon Monoxide.—Ordinary illuminating gas as a source of carbon monoxide has proven entirely satisfactory. It was thought that there might be substances other than carbon monoxide in the gas which might form hemoglobin compounds and interfere with the determination. Accordingly, pure carbon monoxide was prepared by heating oxalic acid with concentrated sulfuric acid and passing the gas produced through sodium hydroxide to free it from carbon dioxide. As far as could be determined on comparison of the two solutions in the colorimeter the colors were identical. Oxyhemoglobin solutions are very unstable. Hence it is necessary, after transferring the blood to the ammonia solution, to saturate with carbon monoxide within an hour. After saturation with carbon monoxide, the solution may, on carefully stoppering and protecting from light, be placed in the ice box and the determination made at leisure. Saturation of the blood should be carried out under a hood. If the laboratory does not possess a hood, the saturation may be accomplished under a funnel, attached to a small water vacuum pump, to remove the gas.

Standard Hemoglobin Solution.—Haldane's standard of a 1 per cent solution of a blood having an oxygen capacity of 18.5 per cent is used. It has been shown by Haldane and Smith (7), Butterfield (8), Barcroft (9), and others, that the oxygen capacity of the blood depends upon its hemoglobin content. A blood of 18.5 per cent oxygen capacity contains approximately 14 gm.

Digitized by Google

hemoglobin per 100 cc. Although the blood of normal men in this country, as shown by Meyer and Butterfield (6), Williamson (10), and also as we have found by use of the method here described, contains on the average 16.6 gm. of hemoglobin, which would correspond to an oxygen capacity of about 22 per cent, it was thought best to keep Haldane's standard for the present. It is a simple matter to compute the gm. of hemoglobin in any given blood from the results obtained.

The standard hemoglobin solution is prepared as follows: A quantity of defibrinated human or ox blood is obtained. The oxygen capacity is determined by the method of Van Slyke (11). The blood may also be standardized by a spectrophotometer or solutions made from hemoglobin crystals prepared in the manner described by Butterfield (8). We have checked several times our standard and found that the oxygen capacity method for standardization is most convenient and satisfactory. The blood is diluted with 0.4 per cent ammonia solution so as to make a 20 per cent solution of a blood with an oxygen capacity of 18.5 per cent. This 20 per cent blood solution is then saturated with carbon monoxide by bubbling through it illuminating gas for 10 minutes. A drop of caprylic alcohol prevents troublesome foaming. The glass tube through which the gas is passed into the blood solution is withdrawn slowly and the bottle stoppered immediately. Rubber corks must not be used in connection with hemoglobin solutions. The cork should be sealed in with paraffin and the solution, protected from light, kept in the ice chest. Such a solution thus protected will keep for months. Several solutions now nearly a year old prepared in this manner have shown no deterioration. 5 cc. of this 20 per cent blood solution made up to 100 cc. with 0.4 cc. of ammonia solution and saturated with carbon monoxide, make the 1 per cent standard for use in the colorimeter and may be prepared from time to time as desired. The 1 per cent standard for routine use may be kept in a dark glass or black painted aspirator bottle, the lower opening of which is provided with a cork, through which passes a glass tube with a ground glass cock for withdrawing small amounts of solution. A glass tube is put through the cork in the top of the bottle and extends to the bottom. Both corks should be sealed with paraffin. This glass tube is connected with an open

Digitized by Google

gas fixture in order that when solution is withdrawn from the bottom, gas rather than air will enter to replace it. Solutions thus prepared may keep for several weeks; but, as a precaution, it is advisable to make fresh 1 per cent solutions frequently; *i.e.*, every 2 or 3 weeks. It should be remembered that dilute hemoglobin solutions are less stable than concentrated solutions; and that hemoglobin solutions keep best in the cold and protected from light. The first indication of solution deterioration is a change in color from the characteristic cherry-red of carbon monoxide hemoglobin to a red with a brownish tinge, due to the formation of methemoglobin.

Comparison in Colorimeters.—The Duboscq or Kober colorimeters have proven to be by far the most accurate and satisfactory instruments for this colorimetric work. Other colorimeters, however, may be used. The difficulties encountered are those inherent in all colorimetric work and in this connection reference to Kober's (12) article may be made. The color of the carbon monoxide hemoglobin, because of the relatively low stimulus threshold for the eye, is admirably suited to colorimeter comparison, slight differences being easily detected. We prefer to use the daylight from a north window. Satisfactory results are, however, obtainable with artificial light when "daylight glass" is used between the source of light and the solution. Considerable experimentation with light filters has failed to improve on the accuracy with which the comparison may be made. No difficulty should be experienced in making the readings check within 0.2 of a single division on the colorimeter scale.

Color comparisons are most accurate when the unknown hemoglobin solution reading falls between 9 and 11 on the colorimeter scale. If the reading of the unknown falls below 8 or above 12, another sample should be taken and the dilution made such that the reading will fall within these limits. This is easily accomplished by varying the amounts of blood and ammonia solution, making the necessary correction in the colorimeter. 2 cc. of solution is adequate for the Kober instrument and 5 cc. for the Duboscq. If a Duboscq or a Kober colorimeter is not available, the Hellige instrument may be used. The 1 per cent hemoglobin standard may be sealed with paraffin into the wedge and the wedge, when not in use, kept in the ice box

Digitized by Google

and protected from light. Attention should be called to the fact that the scales of the Hellige colorimeter are often inaccurately placed. The standard solution must be checked against itself and the scale adjusted so as to read 100 when the color in the cup and wedge match. We have found more difficulty in obtaining accurate checks with this instrument than with the Duboscq or Kober colorimeters; but with care the error should not exceed 2 per cent. 2 cc. of solution is sufficient for the Hellige cup, hence the Sahli pipette, which contains 0.020 cc. of blood, may be used with this colorimeter. As large errors have been found in the calibration of the Sahli pipettes, it is necessary to recalibrate them before using.

Accuracy of the Method.—The accuracy of the method depends to a large extent on the care of the operator in carrying out the various details of the technique. The several steps involved in the method are common chemical procedures with known limits of accuracy. With care duplicate determinations are close and the error should not be more than 1 per cent. A series of ten determinations was made on the same blood. In eight of the ten, the first reading of the colorimeter was in every case exactly the same. In the two remaining a difference of 0.1 of a division on the colorimeter scale occurred.

In the following table are presented a few determinations by the method described using the Duboscq and Hellige colorimeters and comparing with the values estimated from the oxygen

Blood sample.	Hemoglobin determinations.			Difference between Duboscq and oxygen capacity.	Percentage difference.
	Duboscq.	Hellige.	Oxygen capacity.		
	per cent	*per cent*	*per cent*		
1	105.5	104.8	106.5	+1.0	0.94
2	95.2	92.0	94.2	−1.0	1.06
3	128.3	125.0	129.5	+1.2	0.93
4	112.5	115.2	111.7	−0.8	0.68
5	116.2	116.3	116.4	+0.2	0.17
6	79.4	84.0	80.5	+1.1	1.36
7	119.2	115.0	119.4	+0.2	0.17
8	84.0	85.0	83.2	−0.8	0.95
9	82.0	82.0	82.6	+0.6	0.73
10	108.6	—	108.1	−0.5	0.46

Digitized by Google

capacity of the blood. The first ten blood samples of a large series were chosen and illustrate the error in estimation which may be expected. Except in blood Sample 6, no variation between the colorimetric determination (Duboscq) and the oxygen capacity method is greater than 1.0 per cent.

Advantages.—(1) Single determinations may be carried through in 2 minutes. (2) An accuracy within 1 per cent is easily obtained. (3) The standard solution is easily and adequately controlled. (4) Similar solutions are used for comparison, making the color fields, within the limits of the colorimeter, identical. (5) The apparatus required is found in any well equipped laboratory.

SUMMARY.

A method for the determination of hemoglobin colorimetrically with an accuracy of 1 per cent is described.

BIBLIOGRAPHY.

1. Hoppe-Seyler, F., *Z. physiol. Chem.*, 1892, xvi, 505.
2. Haldane, J., *J. Physiol.*, 1900–01, xxvi, 497.
3. Gowers, W. R., *Tr. Clin. Soc., London*, 1879, xii, 64.
4. Sahli, H., Klinische Untersuchungsmethoden, Leipsic and Vienna, 5th edition, 1909, 845.
5. Krogh, M., *Ugesk. Læger*, 1916, lxxviii, 1722.
6. Meyer, E., and Butterfield, E. E., *Arch. Int. Med.*, 1914, xiv, 94.
7. Haldane, J., and Smith, J. L., *J. Physiol.*, 1899–1900, xxv, 331.
8. Butterfield, E. E., *Z. physiol. Chem.*, 1909, lxii, 173.
9. Barcroft, J., The Respiratory Function of the Blood, Cambridge, 1914.
10. Williamson, C. S., *Arch. Int. Med.*, 1916, xviii, 505.
11. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
12. Kober, P. A., *J. Biol. Chem.*, 1917, xxix, 155.

Digitized by Google

GASOMETRIC DETERMINATION OF THE OXYGEN AND HEMOGLOBIN OF BLOOD.

By DONALD D. VAN SLYKE.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 30, 1917.)

The apparatus described in a previous paper[1] for determining the carbonic acid content of plasma may be used with equal facility for determining the oxygen content and the oxygen-binding capacity (hemoglobin) of blood.

For determination of the *oxygen capacity* as a measure of the hemoglobin 3 or more cc. of blood are introduced into a separatory funnel or bottle and distributed in a thin layer about the inner wall, so that maximum contact with the air and complete saturation of the hemoglobin with oxygen are assured. The vessel is rotated for a few minutes so that the blood is kept in a thin layer, or it may be shaken for 15 minutes or longer on a mechanical shaker. The saturated blood is transferred to a heavy test-tube or cylinder.

The blood gas apparatus is now prepared by introducing into it five drops of redistilled caprylic alcohol and 6 cc. of ammonia solution made by diluting 4 cc. of concentrated ammonia to a liter. If saponin powder is available, as much is added to the 6 cc. of ammonia while in the cup of the apparatus as will stick to the end of a glass rod. After the ammonia has been introduced into the 50 cc. chamber of the apparatus the latter is evacuated in the manner described in the previous article, and the air is extracted from the ammonia solution by shaking for about 15 seconds. The extracted air is expelled, and the extraction completed to make sure that no air is left in the solution. Finally, about 2 cc. of the air-free ammonia are forced up into the cup of the apparatus.

The aerated blood is now thoroughly stirred with a rod to

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

Digitized by Google

assure even distribution of the corpuscles, and a sample is drawn into a 2 cc. pipette and run under the ammonia in the cup of the apparatus. (The lower delivery mark of the pipette should be 3 or 4 cm. above the tip. A pipette calibrated for complete delivery would be inconvenient for placing the entire sample of blood below the layer of ammonia.) The blood is now run from the cup into the 50 cc. chamber, the ammonia layer following the blood and washing it in. A few additional drops of ammonia may if necessary be added from a dropper to make the washing complete.

The blood and ammonia in the chamber are mixed and allowed to stand until the blood is *completely laked.* This requires about 30 seconds when saponin is present, 5 minutes when it is not. After laking is complete 0.4 cc. of a saturated potassium ferricyanide solution is introduced to set free the oxygen combined with the hemoglobin. (The cyanide solution is made air-free by boiling or by shaking in an evacuated flask and is kept in a burette under a layer of paraffin oil 2 or 3 cm. thick to exclude air.) The apparatus is now evacuated by lowering the levelling bulb until only a few drops of mercury remain above the lower stopcock, and is shaken, preferably with a rotary motion, to whirl the blood in a thin layer around the wall of the chamber. If the blood was completely laked before the cyanide was added, extraction of the oxygen may be completed by half a minute of efficient shaking. The extracted solution may be drawn into the bulb of the apparatus below the lower cock and the extracted gas measured over mercury as in the determination of carbon dioxide.[2] Or, since the water does not absorb oxygen rapidly enough to cause an error, the solution may be left in the 50 cc. chamber during the reading of the gas volume, the levelling bulb being held at a sufficient height to balance the column of water solution.[2] Finally, in order to make certain that all the oxygen was obtained by the first extraction, the apparatus is evacuated once more and the blood shaken again for a half minute. If the reading shows no increase, it is evidence that all the oxygen was extracted by the first evacuation. If there is an increase the extraction must be repeated again. If the blood is completely

[2] Van Slyke, *J. Biol. Chem.*, 1917, xxx, 353.

Digitized by Google

laked before addition of the ferricyanide, the first shaking practically always removes all the oxygen from the blood solution. Even when laking has been incomplete, however, or the first extraction of the blood otherwise made incomplete, the determination is not lost; for the right result will be obtained if the extraction is repeated until the reading becomes constant.

After each analysis it is well to wash out the 50 cc. chamber of the apparatus with the dilute ammonia solution, as a black precipitate is formed by reaction of the reagents with the mercury. Unless this precipitate is removed it tends to coagulate after a few analyses and interfere with further determinations.

After the blood has been saturated with air the entire procedure above outlined, including the final cleaning of the apparatus, is done in routine determinations in 7 or 8 minutes.

In order to calculate the oxygen bound by the hemoglobin it is necessary to subtract from the gas measured the volume of air physically dissolved by the 2 cc. of blood at atmospheric pressure and the prevailing room temperature. The volume of gas thus corrected may be reduced to standard conditions by multiplying by $(0.999 - 0.0046\ t) \times \frac{\text{barometer}}{760}$, t being the temperature in degrees centigrade. The volumes of air dissolved by the blood at different temperatures are given in Table I. They are calculated in accordance with Bohr's finding, that the solubility of gases in average whole blood is 90 per cent of their solubility in water. The table also gives factors by which one may transpose the readings directly into terms of volume per cent of chemically bound oxygen in the blood, or of per cent hemoglobin on the basis of Haldane's normal average, 18.5 per cent of oxygen in the blood being taken as equivalent to 100 per cent hemoglobin.[3]

Unless one is well experienced with the conditions used for saturating the blood with oxygen, it is advisable, after one portion of a blood sample has been analyzed, to aerate the remainder a second time and repeat the determination, in order to make certain that the hemoglobin of the first portion was completely saturated with oxygen.

[3] Haldane, J., and Smith, J. L., *J. Physiol.*, 1899–1900, xxv, 331.

Digitized by Google

TABLE I.

Factors for Calculating Results from Analysis of 2 Cc. of Blood Saturated with Air.

Temperature.	Air physically dissolved by 2 cc. of blood. Subtract from gas volume read in order to obtain corrected gas volume, representing O_2 set free from hemoglobin.	Factor by which corrected gas volume is multiplied in order to give:	
		Oxygen chemically bound by 100 cc. of blood.	Per cent hemoglobin, calculated on the basis of 18.5 per cent oxygen = 100 per cent hemoglobin.
C.°	*cc.*	*cc.*	*per cent*
15	0.037	$46.5 \times \frac{B}{760}$	$251 \times \frac{B}{760}$
16	0.036	46.3 "	250 "
17	0.036	46.0 "	249 "
18	0.035	45.8 "	247 "
19	0.035	45.6 "	246 "
20	0.034	45.4 "	245 "
21	0.033	45.1 "	244 "
22	0.033	44.9 "	242 "
23	0.032	44.7 "	241 "
24	0.032	44.4 "	240 "
25	0.031	44.2 "	239 "
26	0.030	44.0 "	237 "
27	0.030	43.7 "	236 "
28	0.029	43.5 "	235 "
29	0.029	43.3 "	234 "
30	0.028	43.1 "	233 "

The following example illustrates the calculation:

Observed gas volume, at 20°, 750 mm..... 0.450 cc.
Correction for dissolved air..... 0.034 "
Corrected gas volume..... 0.416 "
$0.416 \times 44.8 = 18.65$ volume per cent oxygen.
$0.416 \times 243 = 101$ per cent hemoglobin.

While the above description and the table are prepared to fit the analysis of 2 cc. samples of blood, which seems the desirable amount for ordinary purposes, either more or less may be taken, the volumes of dilute ammonia and ferricyanide used being changed proportionally. The following data show that good results are obtained with as much as 3 or as little as 1 cc. of blood,

Digitized by Google

although the error must be greater in the latter case because of the small volumes of gas obtained for the final reading.

TABLE II.

Determinations of the Oxygen Capacity of Ox Blood, Using 1 to 3 Cc. Samples.

Volume of blood sample.	Temperature.	Barometer.	Gas volume obtained.	Gas volume corrected for dissolved air.	Volume of O_2 reduced to 0°, 760 mm. bound by 100 cc. of blood.	Per cent hemoglobin, calculated on basis of 18.5 per cent O_2 = 100 per cent hemoglobin.
cc.	*°C.*	*mm.*	*cc.*	*cc.*	*cc.*	*per cent*
3.00	20	752	0.835	0.784	23.45	126.7
2.00	20	752	0.558	0.524	23.49	126.9
1.00	20	752	0.281	0.264	23.67	127.9

For determination of the *oxygen content* of the venous blood as drawn from the body, the preliminary saturation with air is omitted, and precautions, such as are described by Lundsgaard in the accompanying paper,[4] are observed in order to prevent contact of the sample with air. From the point where the blood sample is transferred to the cup of the apparatus for analysis the technique is identical with that described above.

The calculation of the oxygen content is somewhat different from that of the oxygen capacity. The physically dissolved oxygen of venous blood is negligible, and the nitrogen as determined by Bohr is 0.9 volume per cent, reduced to 0°, 760 mm.[5] Therefore the volume of gas obtained in the apparatus is at once multiplied by the factor $(0.999 - 0.0046\ t) \times \frac{\text{barometer}}{760}$ to reduce it to standard conditions. In case 2 cc. of blood have been used, the values of this factor in Column 3 of Table I may be used; in this case they give the content of the blood in chemically bound oxygen plus the dissolved nitrogen gas. The result in cc. of gas per 100 cc. of blood is diminished by 0.9 cc. in order to correct for that amount of nitrogen gas present.

The nature of the results obtained with the method and the degree of accuracy indicated by the agreement of duplicates are shown by Lundsgaard's figures,[4] while Palmer's show the agree-

[4] Lundsgaard, C., *J. Biol. Chem.*, 1918, xxxiii, 133.

[5] Bohr, C., in Nagel, W., Handb. Physiol. Menschen, 1909, i, 117.

Digitized by Google

ment of the gasometric with the colorimetric determination of hemoglobin[6] by the most accurate use of the colorimetric technique. It consequently appears unnecessary to reproduce in this paper further data to illustrate the results obtained with the method.

SUMMARY.

The apparatus previously described for determination of carbon dioxide in blood is used with a similar technique for determination of oxygen. The oxygen is set free from combination with hemoglobin within the apparatus by addition of ferricyanide, is extracted in a Toricellian vacuum, and measured at atmospheric pressure, a few minutes sufficing for an accurate determination.

[6] Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.

Digitized by Google

STUDIES OF OXYGEN IN THE VENOUS BLOOD.

I. TECHNIQUE AND RESULTS ON NORMAL INDIVIDUALS.

By CHRISTEN LUNDSGAARD.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, October 17, 1917.)

The term oxygen content of the venous blood has been used in the literature in two senses. It is applied to the amount of oxygen in the blood in the right heart and also the amount in the venous blood of some organ or group of organs.

In the blood of the right side of the heart the oxygen can be determined by using the lungs plus a bag (1–5) or a part of the lungs (6) as a tonometer. The air in the lungs is brought to equilibrium with the blood gas and a sample of the air is analyzed. In animals, samples of blood can be drawn through a cannula introduced into the heart.

Samples of venous blood from single organs can be obtained in animals when the technical difficulties can be overcome. Extensive studies have been made in the last few years, particularly by Barcroft and his associates (7).[1]

In adults the superficial veins of the limbs and neck, particularly of the arm (vena mediana) are the only sources from which venous blood can be obtained.[2] That means that only blood coming from a limited region, consisting chiefly of muscles, can be studied. Consequently the field of investigation is limited. The conditions can be varied voluntarily only to a small extent. The results have to be interpreted chiefly in an empirical way and by means of general clinical observation. In spite, however, of limitations and *a priori* theoretical difficulties in interpreting

[1] For bibliography see Barcroft (7).

[2] In the Pediatric Department of Johns Hopkins Hospital venous blood is obtained by puncturing the sinus sagittalis in babies. This method makes it possible to obtain venous blood without stasis from children where the superficial veins are too small.

Digitized by Google

the results, the question of the amount of oxygen in the venous blood in health and disease has always been considered important by physiologists and clinicians, although few investigations have been made. Not only are the observations on pathological cases few, but the determinations on normal individuals are even more scarce. The reason for this is probably that we did not have any means of determining blood gases in small quantities of blood until the Haldane-Barcroft method was devised.

Kraus (8) determined the oxygen and CO_2 in the venous blood of a series of normal and pathological cases. Using the old blood-gas pump it was necessary for him to draw large quantities of blood, a circumstance which prevented repeated determinations in the same person. He found in some instances of incompensated heart cases very low values for the venous oxygen compared with that of normal individuals. Using the Haldane-Barcroft method, Morawitz and Röhmer (9) did eighteen determinations on three normal people, twenty determinations on nineteen patients with anemia, and one determination on a patient with polycythemia. In some instances they found an increased consumption,[3] in other instances a normal consumption in their patients. Means and Newburgh (10) using the Haldane-Barcroft method found, like Kraus, low values in four patients with incompensated heart diseases compared with those found in three normal subjects.

We know that in normal individuals the oxygen content of the venous blood is about 13.5 volume per cent, or two-thirds of the total amount in the blood when it is saturated. The values for the oxygen in the blood from an arm vein have not differed materially from the values obtained from the average blood flowing to the right side of the heart. A similar value has been obtained by calculation in the experiments on the blood flow done by Krogh and Lindhard's (11) method.[4] In animals very varying figures have been obtained in blood from different organs (Barcroft, 7).

However, our knowledge has been only approximate. Great variations occur; how great, or how caused, we do not know. It is therefore necessary to establish the limits in not too small a

[3] The term "consumption" means the percentage of oxygen absorbed in the circulation. The arterial blood was considered saturated with oxygen.

[4] See discussion of blood flow experiments in Paper II, *J. Exp. Med.*, 1918, xxvii, in press.

Digitized by Google

number of normal individuals before we can hope to be able to interpret results from abnormal individuals. This paper is a report of thirty-eight determinations on twenty normal individuals.

Technique.

Two operations are involved: first, the drawing of a sample of venous blood; and second, the determination of its oxygen content. The determination of the oxygen has been done by Van Slyke's method (12). This method is quicker, easier to handle, and more exact than the Haldane-Barcroft, where the great volume of foreign air in the apparatus requires elaborate facilities for keeping the temperature constant. A determination with Van Slyke's apparatus (including cleaning of the machine) requires 5 to 10 minutes. The figure can be read with an accuracy of 0.004 cc. Adding to this the inevitable error in measuring and delivering 2 cc. with a pipette, we can say that the difference between duplicates can always be kept within 0.01 cc. of oxygen, which is 0.5 volume per cent of the 2 cc. of blood analyzed. As will be seen from the tables, duplicates usually agree much more closely. Greater differences are caused only by gross errors, such as by imperfect mixture of the corpuscles in the blood, or by allowing time for a slow oxidation (or deoxidation) of the blood between the two determinations (see below).

The other part of the technique, the obtaining of a sample of blood, has been as follows: The arm from which the blood is drawn rests comfortably at the side of the body on a small, moderately soft pillow. The blood is drawn without stasis and the pulse and respiration are counted from the moment the blood appears.[5] The apparatus for blood drawing consists of a very sharp needle connected by means of a 3 to 4 cm. rubber tube to a glass pipette of about 0.5 cm. bore. The pipette and the rubber have a film of sodium oxalate crystals in them. This is obtained by wetting the interior with a saturated solution of oxalate, which is dried with a current of air. In drawing the blood one puts the upper end of the pipette into the mouth (some

[5] It might be more logical to start counting the pulse a half minute before the blood appears. However, the pulse rate usually keeps almost constant under the procedure if it is skilfully done.

Digitized by Google

cotton wool will prevent saliva from flowing down) and quickly introduces the needle in the proximal direction. After a little practice it is generally possible to obtain blood at the first puncture. If the needle is dull, unnecessary pain will be caused and the vein will probably escape the needle. If the point is too long, it will almost invariably hit the opposite wall of the vein before the point is entirely within. The needle is introduced best at an angle of about 50° with the surface of the arm. The opening of the needle must be kept downward or to the side so that the upper wall of the vein will not close the opening when the blood is being drawn. In drawing the blood only a gentle suction is necessary, and if air should happen to be sucked through the blood the sample must be thrown away and a new pipette used. The needle and rubber can often be kept *in situ* while another pipette is connected with the rubber. When a sufficient amount of blood (6 to 12 cc.) has been drawn, the upper end of the pipette is closed by a finger and the needle withdrawn. Then the needle and the rubber are taken away from the pipette and the blood is discharged into a cylinder (2 cm. wide and with a few oxalate crystals in the bottom) below a layer of white mineral oil at least 2 cm. deep. Just before the last 0.5 to 1 cc. of blood has run out, the pipette is again closed with a finger and withdrawn, because the upper part of the blood in the glass pipette has been oxidized and must be discarded. The blood in the cylinder is then stirred up with a glass rod to secure a good mixing with the oxalate. From the cylinder 2 cc. samples of blood are introduced into Van Slyke's apparatus below a layer of evacuated dilute ammonia as described by Van Slyke (12). Before a sample is taken the blood must be stirred up carefully to secure homogeneity. If the blood has been kept in an ice box for some time (see below) it is often difficult to secure homogeneity, even after it has gained room temperature. It gets more sticky and adheres to the sides of the cylinder. It is therefore advisable to do the determinations on fresh blood. After the blood has been stirred with a rod, a 2 cc. pipette is introduced, the upper end being closed to prevent mineral oil from coming up, and after a few seconds' additional stirring with the pipette, the sample is sucked up and discharged into the apparatus. The pipette must be calibrated for 2 cc. outflow, and may be calibrated either for complete discharge or for discharge to a mark

Digitized by Google

on the lower stem. The latter is preferable, but the pipette for complete discharge gives in practice equally good results, because only a very little surface of blood touches the air, so the whole blood column may be used without introducing any appreciable error. The calculation of the amount of oxygen in the sample is simple (see p. 131, Van Slyke's paper (12)). The determination of the total oxygen-combining power of the blood has in some instances been done with the Van Slyke apparatus, in other instances with Palmer's colorimetric method (13). If the blood has been kept in an ice box for some time, it may be impossible to rely upon the homogeneity. Consequently when the main sample of blood is placed under an oil layer, a portion of about 1 or 2 cc. is transferred to a special dish without oil and used for the Palmer determination. It is advisable to have a glass bead in this dish to stir the sample.

RESULTS.

The results are given in Tables I and II. Besides the date and hour of bleeding and of the determination, the pulse and respiration during the bleeding, three groups of figures are given. (1) The oxygen content of the venous blood in volume per cent corrected for temperature and pressure. In all instances double determinations have been done and the average has been taken. (2) The oxygen-combining power of the blood (hemoglobin), in most instances calculated from the determination of the hemoglobin by Palmer's method.[6] (3) The difference between the oxygen in the venous blood and the total oxygen-combining power of the hemoglobin.

This last figure is important. It is difficult to attach much significance to the figure for the venous oxygen, unless we know what percentage of the total oxygen-combining power of the hemoglobin the venous oxygen represents. The venous oxygen uncontrolled by hemoglobin determinations is as incomplete as a nitrogen excretion considered without relationship to the nitrogen intake. We have termed the difference between the venous oxygen and the total oxygen-combining power of the hemoglobin

[6] I am greatly indebted to Dr. Walter W. Palmer, who has done the majority of these determinations.

Digitized by Google

the *oxygen unsaturation* of the venous blood, since it represents that portion of the hemoglobin which does not have its oxygen-binding values saturated. The term unsaturation is used in a sense analogous to that in which it is used in organic chemistry, defined by Webster as "falling short of saturation, not combined to the greatest possible extent."[7]

Thirty-eight determinations on twelve different normal individuals have been done. In Table I are given twenty determinations on one person, and in Table II eighteen determinations on eleven different people. Care has been taken to draw the blood from a person resting as completely as possible, because we know that an increase in the metabolism necessarily is followed by an increased oxygen consumption. Before the drawing of the blood the subjects rested on a couch for some time, in some experiments 10 minutes, in others half an hour. In some instances the blood was taken in the morning, before getting up. It does not seem to make any difference whether the resting period is 10 minutes or 30. The oxygen content of the blood taken in the morning had a tendency to be low, particularly when the person had been awake only a few seconds. The average figure for the oxygen content in the twenty determinations on No. 1 is 13.7 volume per cent, with a maximum of 16.84 volume per cent and a minimum of 9.55. The average figure for the eighteen determinations on the other cases is 13.6 volume per cent, with a maximum of 17.98 and a minimum of 10.36 volume per cent.

The total oxygen-combining power (the hemoglobin) has varied from 21.44 (hemoglobin 116 per cent) to 17.50 (hemoglobin 94.5 per cent). This must, of course, influence the amount of oxygen in the venous blood. In the determination in No. 27, for instance, the amount of oxygen in the venous blood is greater than the total capacity of the hemoglobin in No. 36. Such measures illustrate the necessity of taking as a measure of oxygen

[7] The term oxygen consumption has been applied to the figure for which we prefer the more exact term unsaturation. The use of consumption in this connection implies that all the venous oxygen which the venous blood requires in order to be saturated is lacking for the reason that it has been consumed by the tissues. This assumption would be correct if one could always be certain that the arterial hemoglobin is 100 per cent saturated with oxygen. Since one cannot be certain of this, it seems preferable to use the term unsaturation, which is free from the above assumption.

Digitized by Google

Determination.	Bleeding.			Oxygen content of venous blood.					Hemoglobin (Palmer method).	Calculated oxygen capacity.	Difference between oxygen capacity and oxygen in venous blood = oxygen unsaturation.	Pulse.	Respirations.	Remarks.
				Sample 1.		Sample 2.		Average.						
	Date.	Hour.	Arm.	Hour.	Vol. per cent.	Hour.	Vol. per cent.							
	1917								*per cent*	*vol. per cent*	*vol. per cent*			
1	Jan. 25	3.30	Right.	4.20	14.20	4.50	14.22 14.24	14.22	108	19.96	5.75	78	14	10 min. rest.
2	" 26	3.20	"	3.25	13.95	5.25	14.20 14.62	14.26	—	(19.96)	5.83	74	12	10 " "
3	" 27	4.30	"	4.35	14.50	5.10	14.50	14.50	—	(19.96)	5.42	86	20	10 " "
4	" 29	12.10	Left.	12.30	13.42	12.50	13.42	13.42	—	(19.96)	6.54	70	16	10 " "
5	" 30	4.30	"	4.40	15.40	4.50	15.20	15.30	108	19.96*	4.66	90	14	10 " "
6	" 31	12.20	Right.	12.40	15.80	3.15	15.75	15.78	—	(19.96)	4.18	78	14	10 " "
7	Feb. 6	10.40	"	10.50	16.80	11.10	16.88	16.84	108	19.96	3.12	80	14	10 " "
8	" 6	12.10	"	12.20	12.90	12.35	13.22	13.06	—	(19.96)	6.90	70	14	10 " "
9	" 13	5.10	"	5.15	13.64	5.30	13.64	13.64	110	20.34	6.70	71	14	10 " "
10	" 28	12.20	Left.	12.30	14.25	12.50	14.28	14.27	—	(18.83)	4.56	73	11	½ hr. rest.
11	Mar. 1	11.30	"	11.55	16.15	12.20	16.11	16.13	—	18.83*	2.70	78	12	½ " "
12	Apr. 4	11.00	Right.	12.10	15.06	12.30	14.35	14.70	100	18.50	3.74	72	14	½ " "
13	May 30	7.15	"	7.35	9.54	7.55	9.56	9.55	—	(18.50)	8.95	—	—	Before getting up. Just awakened.
14	" 31	7.15	"	7.30	15.02	7.45	14.99	15.01	100	18.50	3.49	76	14	Before getting up. Had been awake for ½ hour.
15	June 1	7.15	"	7.50	13.97	8.30	14.01	13.99	—	(18.50)	4.51	80	16	Awake for ½ hr.
16	" 2	7.15	"	7.30	12.34	7.45	12.37	12.36	—	(18.50)	6.14	72	14	" " ½ "
17	" 3	7.30	"	7.45	9.79	8.10	10.74	10.27	100	18.50	8.23	66	12	Deep sleep just before blood was taken.
18	" 4	7.15	Left.	7.30	12.54	11.00	12.49	12.52	—	(18.50)	5.98	70	12	Before getting up. Had been awake for ½ hr.
19	" 6	7.15	"	10.15	12.24	11.45	12.20	12.22	102	18.87	6.65	68	12	Awake for ½ hr.
20	" 7	7.15	Right.	7.30	12.23	7.45	11.97	12.10	102	18.87	6.77	70	14	" " ½ "

* Determination of the total oxygen-combining power of the blood done by the Van Slyke apparatus.

Digitized by Google

TABLE II.

Determinations in Venous Arm Blood of Oxygen, Hemoglobin, and Oxygen Unsaturation in Eleven Normal Subjects.

Determination.	Bleeding.			Oxygen content of venous blood.					Hemoglobin (Palmer method).	Calculated total oxygen capacity.	Difference between oxygen capacity and oxygen in venous blood = oxygen unsaturation.	Pulse.	Respirations.	Remarks.	Name.
				Sample 1.		Sample 2.		Average.							
	Date.	Hour.	Arm.	Hour.	Vol. per cent.	Hour.	Vol. per cent.								
	1917								*per cent*	*vol. per cent*	*vol. per cent*				
							15.20								
21	Feb. 1	12.10	Right.	12.20	15.50	12.40	15.42	15.37	115	21.26	5.89	70	14	10 min rest.	
22	" 7	11.40	Left.	11.50	16.03	12.10	16.24	16.14	116	21.44	5.30	84	13	10 " "	Dr. St.
23	" 7	12.40	"	12.50	16.76	1.10	16.66	16.71	116	21.44	4.73	81	14	10 " "	
24	Feb. 8	12.15	Left.	12.25	11.66	3.00	11.98	11.82	—	17.48*	5.66	55	12	10 min. rest.	
25	Apr. 9	12.00	"	3.20	12.27	3.30	12.25	12.26	99	18.48	6.22	68	—	10 " "	Dr. F.
26	" 30	12.00	Right.	12.30	13.17	1.00	13.04	13.11	100	18.50	5.39	58	19	½ hr. rest.	
27	Feb. 5	11.20	Left.	11.40	18.01	11.55	17.95	17.98	113	21.11*	3.13	68	14	10 min. rest.	
28	" 5	12.40	"	12.50	15.82	1.10	16.25	16.04	—	—	5.07	—	—	10 " "	Dr. V. S.
							14.04								
29	Feb. 9	10.00	Right.	10.10	13.93	10.25	14.15	14.04	101	18.86	4.82	78	14	10 min. rest.	
30	" 9	1.15	"	1.30	12.35	2.10	12.61	12.48	—	18.86	6.38	76	14	10 " "	Dr. Bl.

Digitized by Google

31	June 3	11.15	Left.	11.30	12.00	12.15	12.00	12.00	108	20.00	8.00	54	22	½ hr. rest.	
32	" 14	3.30	"	3.50	10.62	4.10	10.68	10.65	98.4	18.30	7.65	52	20	½ " "	Dr. L.
33	Feb. 2	10.45	Right.	10.55	13.15	11.10	13.10	13.13	107	19.80*	6.67	70	18	10 min. rest.	T
34	Feb. 1	4.20	Left.	4.30	15.55	4.45	15.45	15.50	100	18.50	3.00	88	18	10 min. rest.	R
35	May 17	10.30	Right.	11.00	12.48	11.30	12.74	12.61	105	19.40	6.79	62	24	½ hr. rest.	Dr. D.
36	June 6	10.40	Left.	11.00	10.75	11.30	11.15	10.95	94.5	17.50	6.55	70	—	½ hr. rest.	Miss S.
37	June 8	7.15	Left.	7.30	10.40	7.45	10.32	10.36	103	19.04	8.68	78	—	Before getting up.	Dr. P.
38	June 13	7.00	Left.	7.15	11.52	7.25	11.56	11.54	110	20.37	8.83	70	14	Before getting up.	Dr. M.

* Determination of the total oxygen-combining power of the blood done by the Van Slyke apparatus.

Digitized by Google

TABLE III.

Determinations of Oxygen in Venous Blood Kept for Varying Periods under a Layer of Mineral Oil.

Name.	No. in Tables I and II.	Bleeding.		Determination.		Oxygen content of venous blood.	Remarks.
		Date.	Hour.	Date.	Hour.		
		1917		*1917*		*vol. per cent*	
L.	2	Jan. 26	3.20 p.m.	Jan. 26	3.25 p.m.	13.95	5 min. in laboratory.
				" 26	5.25 "	14.24	3 hrs. in refrigerator at 6°.
				" 27	12 n.	14.20	18½ " " " " 6°.
				" 27	3.30 p.m.	12.28	3½ " ". laboratory " 24°.
L.	3	Feb. 27	4.30 p.m.	Feb. 27	4.35 p.m.	14.50	5 min. in laboratory at 24°.
				" 27	5.10 "	14.50	35 " " " " 24°.
				" 28	10.30 a.m.	14.60	41 hrs. in refrigerator " 6°.
L.	6	Jan. 31	12.20 p.m.	Jan. 31	12.40 p.m.	15.80	20 min. in laboratory at 23°.
				" 31	3.15 "	15.75	2½ hrs. " " " 23°.
W.	(2) From another publication.	Mar. 24	12 n.	Mar. 24	2.30 p.m.	7.68	2½ hrs. in laboratory at 23°.
				" 24	2.50 "	7.66	20 min. " " " 23°.
				" 27	3.40 "	11.11	73 hrs. in refrigerator " 6°.
L.	(1) From another publication.	July 2	2.00 p.m.	July 2	2.10 p.m.	17.14	10 min. in laboratory at 25°.
				" 2	2.30 "	17.32	10 " " " " 25°.
				" 9	3.00 "	19.05	7 days in ice box.
L.	(2) From another publication.	July 3	9.50 a.m.	July 3	10.10 a.m.	17.63	20 min. in laboratory at 26°.
				" 3	10.30 "	17.86	20 " " " " 26°.
				" 7	2.00 p.m.	18.64	4 days in ice box.
T.	(4) From another publica-	July 13	9.30 a.m.	July 13	11.10 a.m.	13.83	1½ hrs. in laboratory at 27°.
				" 13	3.40 p.m.	13.99	4½ " " ice box.
				" 13	9.30 "	13.82	5 " " laboratory.

Digitized by Google

consumption the oxygen unsaturation rather than the oxygen content of the venous blood. The average value of the oxygen unsaturation for Case 1 is 5.5 volume per cent, the minimum being 2.70, the maximum 8.95. The average for the eighteen determinations on Cases 2 to 12 is 6.0 volume per cent, with a minimum of 3.00, a maximum of 8.83 (Table IV).

The determinations are too few to allow an interpretation of the different causes of the variations.[8] Series of determinations on different individuals under different conditions might throw light upon that problem. There is generally a decrease in the oxygen unsaturation with increasing pulse rate, but it is by no means invariable. It is worth mentioning that a value for the oxygen unsaturation of more than 8 volume per cent is found in only four instances, in all of which the blood was drawn in the morning, a few seconds after the subject awoke. In other words, the highest degree of unsaturation is found under circumstances where the metabolism is lowest, when the individual usually has his lowest pulse rate, and when all exciting impressions have been excluded for a considerable time.

In some instances it has been impossible to do the blood analysis immediately after a bleeding. In order to find out how long, and under what conditions the blood samples can be stored, experiments have been done as shown in Table III.

The figures show that the blood can be kept in an ice box at a low temperature (6°C.) for a considerable length of time (at least 24 hours) before any appreciable changes take place. After a certain time the blood will absorb oxygen through the oil and the values will increase. The opposite happens when the blood is kept in the laboratory. We cannot expect it to keep constant more than 2 hours. After that interval the oxygen content diminished rapidly, probably on account of bacterial action.

[8] A series of papers in which the technique described in this paper is applied to the clinical study of patients with heart disease will be published in *The Journal of Experimental Medicine.*

Digitized by Google

SUMMARY.

1. A report is made of a series of determinations by the Van Slyke method of the oxygen in the blood drawn from the vena mediana in normal resting individuals.

2. A procedure for drawing the blood without stasis or absorption of air has been devised.

3. The difference between the total oxygen-combining power of the hemoglobin and the oxygen in the venous blood is calculated. This figure is termed the oxygen unsaturation of the venous blood.

4. The results are the following:

TABLE IV.

No. of individuals.	No. of determinations.	Oxygen content of venous blood. Vol. per cent.			Oxygen unsaturation of venous blood. Vol. per cent.		
		Maximum.	Minimum.	Average.	Maximum.	Minimum.	Average.
12	38	17.98	9.55	13.6	8.95	2.70	5.8

BIBLIOGRAPHY.

1. Plesch, J., *Z. exp. Path. u. Therap.*, 1909, vi, 380.
2. Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244.
3. Fridericia, L. S., *Danske Vidsk. Selskabs Oversigt*, 1916, 113.
4. Boothby, W. M., and Sandiford, I., *Am. J. Physiol.*, 1916, xl, 547.
5. Bornstein, A., *Arch. ges. Physiol.*, 1910, cxxxii, 307; *Z. exp. Path. u. Therap.*, 1913, xiv, 135.
6. Loewy, A., and von Schrötter, H., *Z. exp. Path. u. Therap.*, 1905, i, 279.
7. Barcroft, J., The Respiratory Function of the Blood, Cambridge, 1914, Bibliography.
8. Kraus, F., Die Ermüdung als ein Mass der Constitution, Cassel, 1897, iii.
9. Morawitz, P., and Röhmer, W., *Deutsch. Arch. klin. Med.*, 1908, xciv, 529.
10. Means, J. H., and Newburgh, L. H., *Tr. Assn. Am. Phys.*, 1915, xxx, 51.
11. Krogh, A., and Lindhard, J., *Skand. Arch. Physiol.*, 1912, xxvii, 100.
12. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
13. Palmer, W. W., *Proc. Soc. Exp. Biol. and Med.*, 1916-17, xiv, 175.

Digitized by Google

ADENINE AND GUANINE IN COW'S MILK.

By CARL VOEGTLIN and CARL P. SHERWIN.

(*From the Division of Pharmacology, Hygienic Laboratory, Washington.*)

(Received for publication, November 19, 1917.)

In the course of some work on the isolation of the antineuritic substance present in cow's milk, we incidentally discovered that this food contains fair amounts of adenine and guanine. Inasmuch as we were unable to locate any reference in the literature to such findings, we present this brief note, which may be of interest in connection with the biochemistry of milk for the following reasons: (1) milk is usually considered to be practically purine-free and has been widely used in metabolism experiments as a purine-free diet; (2) milk was used as a diet in experiments to determine whether or not the animal body can synthesize purines and nucleic acid from purine-free food; (3) the mammary glands may act under certain conditions as an excretory organ for metabolism products, drugs, and poisons.

Concerning the occurrence of purines in cow's milk, Schmidt-Mülheim (1883) claims that he detected free hypoxanthine. Burián and Schur (1897) state that they did not succeed in isolating uric acid from cow's milk; however, the presence of xanthine bases was established by means of the silver precipitate method. The following figures are given: 1 liter of cow's milk yielded approximately 0.005 gm. of purine nitrogen in the silver purine precipitate.

One grave objection can be raised to the method employed by these investigators; namely, the use of $AgNO_3$, a reagent which is known to deaminize amino-purine unless it is used carefully. It is possible that the hypoxanthine which was isolated was in reality due to the deamination of adenine primarily contained in the milk.

Digitized by Google

EXPERIMENTAL.

The milk was obtained from a herd of Jersey cows and represented a mixed sample. 100 liters of milk were worked up in the following manner: The whole milk, after a thorough shaking, was made slightly acid with acetic acid. The curdled milk was then filtered by suction. To the filtrate an equal volume of 95 per cent alcohol was added and again filtered. This seemed to remove most of the remaining protein and a clear transparent liquid was the result of this last operation. The addition of the alcohol also served a second purpose. As the fluid had to be evaporated to a very small volume *in vacuo*, the addition of alcohol lowered the boiling point of the solution, thus preventing overheating. After evaporating the solution at 45°C. *in vacuo* to about 5 per cent of its original volume a thick white liquid remained. This residue contained fats, milk sugar, lipoids, traces of protein, as well as inorganic salts. The fats and lipoids were removed by repeated extraction with ether, and the protein was removed by a single filtration. The remaining liquid was acid in reaction (acetic), yellowish green in color, and had a syrupy consistency. After standing for 24 to 48 hours, crystals began to appear and after about 2 weeks (depending on concentration) the crystallization became complete. The crystals were lactose. For the sake of convenience 8 liters of milk were worked up in this manner daily. The final volume of each lot of milk amounted to from 350 to 400 cc. The various portions were united and treated with a concentrated aqueous solution of either silver acetate or nitrate. One-fourth of the entire solution (1,500 cc.) was thus treated with Ag acetate in excess. The remainder was precipitated with an excess of silver nitrate. The precipitate caused by the addition of the silver solutions to the milk residue was necessarily divided into two lots; silver purine precipitate from $AgNO_3$ and silver purine precipitate from silver acetate. Both precipitates were filtered off separately and washed well with cold water.

Treatment of Silver Purine Fraction.

The $AgNO_3$ purine precipitate was washed off the filter, suspended in water, to which sufficient HCl had been added to

Digitized by Google

transform all the silver into AgCl. The latter was filtered off and the filtrate was carefully evaporated to a small volume *in vacuo* at low temperature in order to protect the aminopurines from the destructive action of the nitric acid. The remaining solution was made alkaline with ammonia in order to precipitate the guanine. The guanine settled out as a gelatinous mass, which was redissolved in dilute HCl and reprecipitated with NH_4OH. After drying this precipitate, an amorphous white mass remained.

The filtrate from the original guanine precipitate was treated with CO_2 in order to remove the adenine. This method did not prove satisfactory, so the solution was slightly acidified with HCl and treated with a saturated solution of picric acid in water. After some standing the resulting yellowish brown precipitate was filtered off and recrystallized from water. It gave all the tests for adenine picrate.

The silver acetate purine fraction was treated in the same way and corresponding amounts of guanine and adenine picrate were obtained.

The total amount of adenine picrate from both fractions after recrystallization amounted to 1.141 gm. (0.72 gm. from $AgNO_3$; 0.421 gm. from Ag acetate) or 0.498 gm. of adenine. The total guanine collected (recrystallized) was 1.09 gm.

Identification of Guanine.

The guanine recovered from the purine fraction after reprecipitation from acid solution by NH_3 and drying was a white amorphous powder. It was converted into its different salts as follows.

Guanine Sulfate.—A fraction of a gram of the powder was dissolved in dilute H_2SO_4 (warm) and allowed to stand over night on ice. The following morning long white rhombic needles had formed. The crystals were filtered off, dried *in vacuo* to constant weight, and then a melting point was taken. The crystals lose their water of crystallization at 118–120°. No definite melting point was obtained.

Guanine Picrate.—0.3 gm. of guanine was dissolved in dilute HCl and treated with a saturated aqueous solution of picric acid.

Digitized by Google

A substance crystallized out in the form of clusters of needles, only light yellow in color. The crystals were filtered off and dried at 100°. The substance had no true melting point but decomposed at 190–200°.

0.1509 gm. of the picrate yielded on analysis 0.0422 gm. N or 29.29 per cent. Guanine picrate contains 29.41 per cent N.

Guanine Hydrochloride.—A small amount of the guanine obtained from milk was dissolved in dilute HCl. On standing at a low temperature crystals formed in the shape of needles. These were filtered off and dried as well as possible in the desiccator over concentrated H_2SO_4. The substance did not have a definite melting point.

0.1212 gm. of the substance yielded 0.0457 gm. N or 37.52 per cent. N calculated for guanine HCl = 37.26 per cent.

Identification of Adenine.

Adenine Picrate.—Adenine picrate as obtained in the way previously described was dried at 105° and gave a melting point of 278–281°.

0.0891 gm. of the picrate yielded 0.0276 gm. N or 30.98 per cent. N calculated for adenine picrate = 30.82 per cent.

The picrate was sparingly soluble in hot water, practically insoluble in cold water. It crystallized in yellow needle-form.

Recovery of Adenine from Adenine Picrate.

From the adenine picrate the free adenine was obtained by treating the picrate with HCl and toluene-ether. The major portion was lost in this operation, but a small percentage of the adenine reclaimed had the following properties.

Melting point, 350–360°; Kjeldahl nitrogen, 0.0722 gm. Adenine gave 0.0372 gm. N or 51.52 per cent; N calculated for adenine = 51.85 per cent. The substance was recovered in the form of white needles.

Digitized by Google

SUMMARY AND DISCUSSION.

As will be seen from the data reported, cow's milk contains small amounts of both guanine and adenine. 1 liter of milk contains at least 5 mg. of adenine and about 10 mg. of guanine. These values may be considered as minimum values, as the method of isolation of these amino-purines is by no means quantitative. It is interesting to compare these figures with those of Krüger and Salomon (1898–99) dealing with the adenine and guanine content of normal human urine. These authors found in 10,000 liters of urine 3.54 gm. of adenine, no guanine, 10.11 gm. of xanthine and 8.50 of hypoxanthine (accordingly, 1 liter of urine contains 0.35 mg. of adenine and no guanine). The question as to whether the purines found in milk are derived from the blood purines or whether they are formed from the breaking down of the nucleic acid in the mammary gland is still left open.

BIBLIOGRAPHY.

Schmidt-Mülheim, *Arch. ges. Physiol.*, 1883, xxx, 379.
Burián, R., and Schur, H., *Z. physiol. Chem.*, 1897, xxiii, 60.
Krüger, M., and Salomon, G., *Z. physiol. Chem.*, 1898, xxiv, 364; 1898–99, xxvi, 350.

Digitized by Google

Digitized by Google

THE NUTRITIVE PROPERTIES OF KAFIRIN.

By ALBERT G. HOGAN.

(From the Department of Chemistry, Kansas State Agricultural Experiment Station, Manhattan.)

(Received for publication, November 22, 1917.)

Agriculturists have known for years that the sorghum grains are peculiarly adapted to regions of light or insufficient rainfall, and for that reason they have urged that larger areas in the semi-arid regions be devoted to the culture of kafir, feterita, and milo. In view of the drouth-resisting properties of these grains, and their increasing economic importance, it seemed desirable to investigate more closely their nutritive value.

Such knowledge as we have of the individual proteins of kafir is largely due to Johns and Brewster (1916). These authors found that most of the nitrogenous matter of kafir, about 67 per cent of the total protein, was soluble in alcohol, and they coined the term *kafirin* for this particular protein. They made a careful chemical examination of the material, and for convenience, some of their results are included below.

Analysis of an average of thirteen samples of kafirin gave: C, 55.19 per cent; H, 7.36; N, 16.44; S, 0.60; O, 20.41. The authors also determined the percentage of diamino-acids in kafirin by the Van Slyke method, with the following results: arginine, 1.58 per cent; lysine, 0.90; histidine, 1.00; and tryptophane, present.

Because kafirin forms such a large proportion of the protein of kafir, its nutritive properties were studied in some detail.

A basal ration[1] was prepared in which, for all practical purposes,

	12 per cent kafirin.	15 per cent kafirin.
	gm.	*gm.*
Protein	126	157
Protein-free milk	257	257
Butter	300	300
Starch	295	265
Agar	20	20

Digitized by Google

kafirin formed the sole source of protein supply. In all cases this diet resulted in nutritive failure (Chart 1), and accordingly kafirin was supplemented with other deficient proteins, gliadin and gelatin, in the hope that some clue might be obtained to the amino-acid deficiencies of kafirin. These proteins were added to the basal ration, in the proportion of 100 gm. supplement to each 1,000 gm. of the ration. The addition of gliadin, for a short time at least, barely sufficed to maintain the live weight of the animals; but the addition of gelatin enabled them to grow slowly. Both proteins were then added to the basal ration (Chart 2) in the proportion of 50 gm. of each to 1,000 gm. of the ration, with the result that apparently the animals grew still more rapidly than they did when gelatin alone was added. The data are illustrated graphically in Charts 1 and 2.

It is evident, taking the period as a whole, that the rats receiving both protein supplements grew more rapidly than the rats receiving only gelatin. Isolated periods could be selected in which the animals of this latter lot grew as rapidly as the others, but probably no especial significance should be attached to that point. Two or three obvious explanations for the behavior of these rats could be advanced, but the theory chosen tentatively was that there are at least two limiting factors in kafirin as a source of protein. Gelatin supplies one, and gliadin the other.

The most conspicuous advantage of gelatin over gliadin as a source of amino-acid supply is its much larger percentage of lysine. In other respects, however, gelatin is decidedly deficient, as it lacks tyrosine, cystine, and tryptophane. Gliadin, on the other hand, is deficient in lysine, but contains the other three amino-acids in fairly satisfactory proportions. It seemed logical therefore to assume that lysine is the first limiting factor in kafirin, and that the second is one of the three other amino-acids mentioned, tyrosine, cystine, or tryptophane.

Some of the earlier work concerning the rôle of tryptophane in nutrition has been summarized in an earlier paper (1917) from this laboratory. The important rôle that cystine plays in nutrition has been demonstrated by Osborne and Mendel (1915). Their data show that when casein is the only protein in the ration cystine under certain conditions becomes a limiting factor. According to Mathews (1915) casein contains 0.8 per cent of sulfur,

Digitized by Google

while according to Johns and Brewster (1916) kafirin contains only 0.6 per cent of that element. It is an assumption to calculate that all the sulfur of these proteins is cystine sulfur, but the data make it seem possible that the amount of cystine in kafirin is well below the optimum.

The statement is frequently encountered in the literature that tyrosine is an essential amino-acid. Totani (1916) believes that tyrosine is not necessarily essential, and apparently the facts in the case have not been finally established.

In an effort to investigate the deficiencies of kafirin more definitely, the amino-acids[2] mentioned were added directly to the basal rations. These additions were made singly or collectively as follows: lysine, cystine, lysine plus cystine, lysine plus tyrosine, lysine plus tryptophane.

The lot receiving the basal ration plus lysine grew slowly, in accordance with our expectations. Evidently lysine (Chart 2) is the first limiting factor in kafirin. Accordingly, in our other feeding trials (Chart 3), lysine was always made a part of the basal ration. The further addition of tryptophane to the diet did not increase the rate of growth, and a similar addition of tyrosine (Chart 4) was equally ineffective. When cystine was superimposed on this ration, however, the animals grew somewhat more rapidly. Small variations might seem insufficient to establish this point, but the unanimity of the results seems conclusive. Furthermore, the marked acceleration in growth that follows the addition of cystine after it has been withheld for some time seems even more conclusive. Accordingly, our interpretation of the data is that cystine is the second limiting factor in kafirin. Charts 3 and 4 give the data in greater detail.

[2] In some cases the tyrosine used was a Kahlbaum preparation, in others it was prepared by the author. The cystine, tryptophane, and lysine were prepared in this laboratory.

The amount of lysine added varied from 3 to 6 per cent of the kafirin in the ration, but the larger amount seemed no more effective than the smaller. The cystine formed 5 per cent of the kafirin, and enough tyrosine was added to equal 4 per cent of the protein.

Digitized by Google

The Lysine Requirement of Immature Animals.

The data bring out one fact of general application; namely, that lysine is indispensable, even for the maintenance of young animals. This point has been in doubt for some time because of the difficulty in proving that a diet contains no lysine. Gliadin was formerly thought to lack that particular amino-acid, and because of that belief, earlier experimental work on the nutritive properties of gliadin was assumed to have special significance. Osborne and Mendel (1914) stated that rats maintained their live weight unchanged, but failed to grow when gliadin formed the sole source of protein in the ration. Their interpretation was that lysine is necessary for growth, but is not necessary for maintenance. When somewhat later the presence of lysine in gliadin was established, they (1916) recognized the element of doubt attached to their earlier pronouncement. The behavior of animals receiving their protein in the form of zein has also been interpreted as an indication that lysine is not required for the maintenance of nutritive equilibrium. Willcock and Hopkins (1906) found that mice receiving zein as the sole source of protein died in less than 2 weeks. Those receiving zein and tryptophane were active after 16 days of zein feeding, but had lost weight. Wheeler (1913) obtained similar results, but even after fortifying the zein with tryptophane the animals constantly lost weight. Similar results are recorded by Osborne and Mendel (1914) in their series of experiments. Their animals receiving a zein plus tryptophane diet were at a nutritive standstill or lost weight, though they grew rather rapidly on the further addition of lysine.

The precise status of lysine in nutrition remained uncertain, however, because of the difficulty in estimating or even detecting small quantities of that amino-acid. Recently Osborne, Van Slyke, and collaborators (1915) published data indicating no inconsiderable quantity of lysine in gliadin. The method of Van Slyke yielded an average result of 1.33 per cent, and the direct method of Kossel and Kutscher resulted in the recovery of 0.64 per cent of the gliadin as lysine. There is no doubt, therefore, that gliadin contains approximately 1 per cent of lysine, while kafirin, according to Johns and Brewster (1916),

Digitized by Google

contains 0.90 per cent. On the other hand, such evidence as is available points strongly to the complete absence of lysine from zein. Osborne and Leavenworth (1913) found no trace. Furthermore, according to a theory advanced by Van Slyke and Birchard (1914), proteins containing lysine possess free amino groups that react with nitrous acid; but zein gives no evidence of such a reaction. Gliadin, on the contrary, according to their data yields 1.10 per cent of the total nitrogen as free amino nitrogen.

Although this evidence may be questioned, yet such weight as it has is all against the presence of lysine in the zein complex. If the facts deduced from zein feeding contradict our findings while feeding kafirin, we have no explanation for the discrepancy.

SUMMARY.

Lysine is the first limiting factor in kafirin and cystine is the second.

Lysine is indispensable for the maintenance of young animals.

BIBLIOGRAPHY.

Hogan, A. G., *J. Biol. Chem.*, 1917, xxix, 485.
Johns, C. O., and Brewster, J. F., *J. Biol. Chem.*, 1916–17, xxviii, 59.
Mathews, A. P., Physiological Chemistry, New York, 1915, 110.
Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1913, xiv, 481.
Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325.
Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351.
Osborne and Mendel, *J. Biol. Chem.*, 1916, xxv, 1.
Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 259.
Totani, G., *Biochem. J.*, 1916, x, 382.
Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539.
Wheeler, R., *J. Exp. Zool.*, 1913, xv, 209.
Willcock, E. G., and Hopkins, F. G., *J. Physiol.*, 1906–07, xxxv, 88.

Digitized by Google

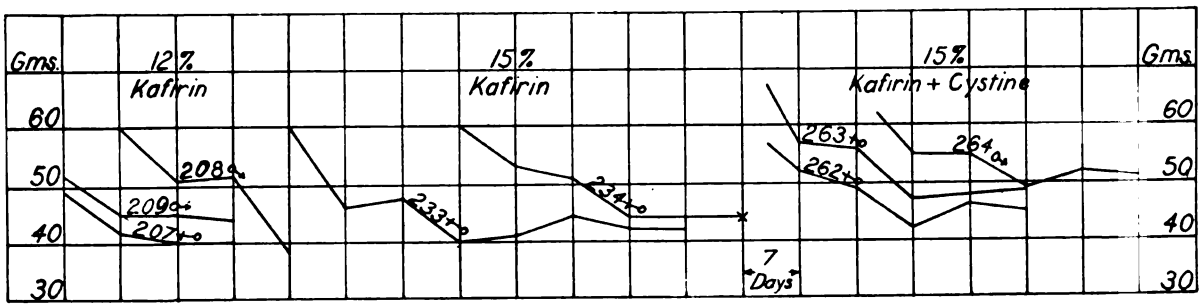


CHART 1. One lot of animals received 12 per cent, another 15 per cent kafirin.[1] The inadequacy of this protein is indicated by the behavior of these rats. They lost weight and manifested the usual symptoms of malnutrition, such as diseased eyes, roughened coat, and muscular weakness. Rat 234 died at the point indicated by X. The lot receiving cystine as a supplement is included here inasmuch as these animals had obviously not been affected in any way by that addition to the ration.

Digitized by Google

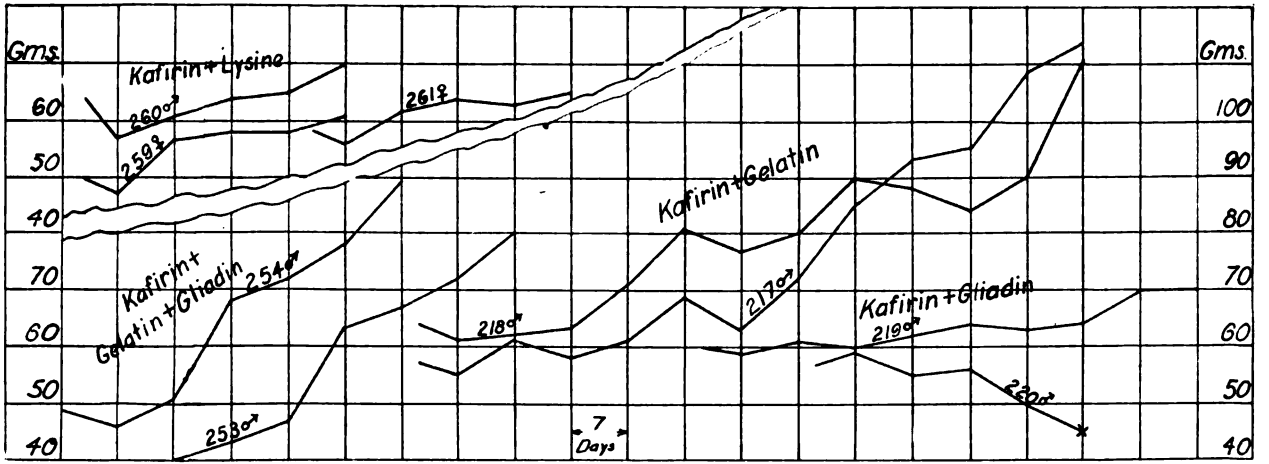


CHART 2. All animals received the same basal ration,[1] 15 per cent kafirin. The addition of gelatin as a supplement (10 per cent of the entire ration) permitted fairly rapid growth. Gliadin in equal quantity was much less effective. One rat receiving this adjuvant grew very slowly, the other died at the point indicated by X. When the gelatin and gliadin diets were combined in equal proportions and fed to another group of rats, growth occurred more rapidly than on the larger quantity of gelatin given alone.

These facts seemed to indicate that lysine is the first limiting factor in kafirin, and when this amino-acid (5 per cent of the protein) was added to the basal ration, that inadequate diet was converted into one permitting a slow rate of growth.

Digitized by Google

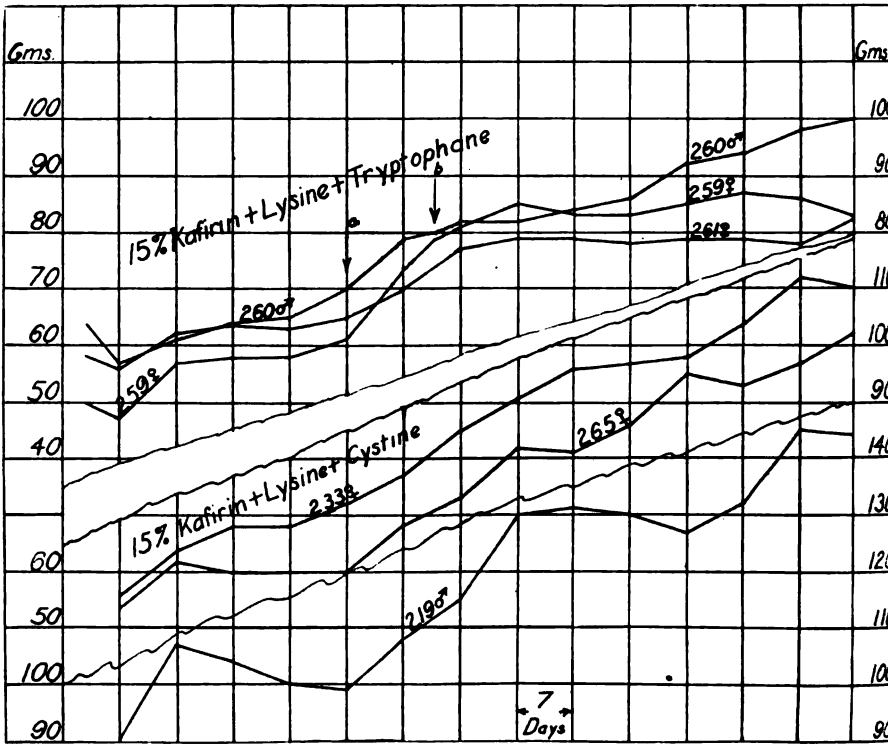


CHART 3. All these animals received a ration containing 15 per cent kafirin,[1] and lysine. Three of them, Rats 259, 260, and 261, received in addition tryptophane and cystine at the point shown by *a*. At point *b*, the cystine feeding was discontinued and thereafter these rats received as amino-acid supplements only lysine and tryptophane. The other three animals, Rats 219, 233, and 265, received cystine in addition to the lysine at all times.

The effects of cystine feeding are not marked, but the lot receiving this amino-acid made a distinctly greater gain than the other. The males normally grow faster than the females, yet Rat 260 on the tryptophane ration, a male, grew somewhat more slowly than either of the females receiving the cystine diet. For a short time, the tryptophane lot received cystine, and it seems significant that in this brief period they should exhibit such a marked increase in the rate of growth. If the cystine feeding had been omitted, it seems probable that these rats would have made a still more unfavorable showing as compared with the cystine lot.

Digitized by Google

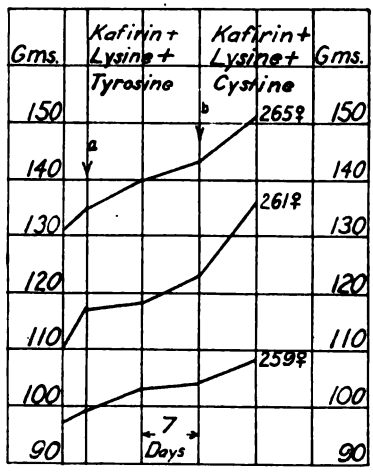


CHART 4. The data shown in this chart are not complete, as the investigation was unavoidably interrupted. In spite of the fact that the period of these feeding trials was short, however, we believe that it was sufficiently long, at least when considered in relation to Chart 3, to admit of a final conclusion.

Rats 259 and 261 had been receiving 15 per cent kafirin and lysine[2] plus tryptophane,[2] and Rat 265 had been on a diet of 15 per cent kafirin plus lysine plus cystine.[2] At the point indicated by *a* all were changed to a ration of 15 per cent kafirin plus lysine plus tyrosine.[2] The rate of growth of the animals was apparently not affected, certainly it was not increased. At point *b* tyrosine was replaced by cystine, and the rate of gain was increased in all cases. In two instances it was marked.

Digitized by Google

Digitized by Google

THE OCCURRENCE OF LICHENASE IN THE DIGESTIVE TRACT OF INVERTEBRATES.*

By MINNA E. JEWELL and HOWARD B. LEWIS.

(*From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.*)

(Received for publication, November 19, 1917.)

No animal enzyme has yet been found by which the hydrolysis of lichenin, the peculiar dextran of *Cetraria islandica*, may be accomplished. Of the vegetable enzymes, Saiki (1) has reported that the taka-diastase of *Eurotium oryzæ* and the inulase of *Aspergillus niger* were active in the hydrolysis of lichenin, while malt diastase was inactive, as previously observed by Brown (2). Examination of human saliva (1, 2, 3) and gastric juice (3), of extracts of the pancreas of the dog (1, 3) and ox (3), and of the intestine of the pig (1) for the presence of a lichenase have uniformly yielded negative results. In view of this resistance to hydrolysis by the body enzymes of the higher animals, as pointed out by Nilson (3), the problem of the utilization of lichens by animals, such as the reindeer, and in human dietaries offers many difficulties. Bacterial decomposition might be expected to play a rôle here, but according to Saiki (1) cultures of *Bacillus coli* do not form reducing sugars from lichenin.

In connection with a study of the fresh water crayfish, it was observed that extracts of the hepatopancreas possessed marked hydrolytic properties towards solutions of lichenin, and further investigations of certain other invertebrates disclosed the existence of lichenase in these animals. Accordingly, a systematic search for the presence of lichenase in the alimentary tract of such vertebrate and invertebrate forms as were available was undertaken.

The lichenin used as a substrate was derived from three sources: (1) crude extracts of Iceland moss, (2) lichenin obtained from

* A preliminary account of this investigation was presented before the Society for Experimental Biology and Medicine, New York, Dec. 20, 1916.

Digitized by Google

a chemical supply house, and (3) lichenin prepared and purified in this laboratory by the method of Berg (4). The first preparation was used only in comparison with one of the other preparations. Tests for isolichenin made on the other two preparations were uniformly negative (color with iodine). No differences were noted in these two preparations when used as substrates in the enzyme experiments. As controls of the activity of the extracts under investigation, freedom from bacterial decomposition, etc., tests were also carried out on other typical carbohydrates, inulin, raffinose, sucrose, and starch, for the presence of other enzymes. In view of the widespread distribution of amylase, it was thought that the absence of amylase would be indicative of loss of enzyme activity in the preparation of the extract. As an additional control, the tissue extract itself was incubated with an amount of water equal to the volume of the carbohydrate solution used. This was done in order to test for the presence of any polysaccharide (*e.g.*, glycogen) which might be present in the tissue and yield a reducing sugar on autolysis. Only in the case of two of the mollusks studied (*Unio gibbosus* and *Lampsilis luteola*) was there found evidence of the presence of such a carbohydrate. In the experiments with these species comparative tests showed the presence of enzymes which acted upon the other sugars also. Control tests with boiled extracts were also made in each case. All experiments were carried out at room temperature, at about 21°C. Toluene was used as a preservative. 0.5 per cent solutions of lichenin and 1 per cent solutions of the other carbohydrates were tested. Tests for reducing sugars[1] with Fehling's solution were made after 1, 3, 5, and 7 days.

The hepatopancreas was chosen for investigation in most of the invertebrates studied (Nos. 4 to 18, Table I). In the small animals (1, 3, 20, Table I) the entire organism was used, while in the earthworm and grasshopper the entire alimentary canal was removed for examination. The animals were killed with ether, the tissues removed immediately, washed, and ground up

[1] In a number of experiments the reducing sugar was further identified by the formation of the osazone. An osazone crystallizing from the hot solution, and identified microscopically as glucosazone, was obtained from the tubes showing reduction, but never from the control tubes.

Digitized by Google

with sand and water in a mortar. The extracts were then filtered through cotton. Extracts of the organs of the pig, sheep, and dog could not be made until from 12 to 24 hours after their removal from the body.

The detailed results are shown in the tables. *An enzyme capable of hydrolyzing lichenin with the formation of a reducing sugar was found to be present in the alimentary system of each of the twenty species of invertebrates examined (Table I), while in no case was such an enzyme observed in the alimentary tract of the vertebrates under investigation (Table II).* In every instance the extracts of the vertebrate tissue were active, as evidenced by their strong amylolytic properties. This inactivity of extracts of the organs of the vertebrate alimentary system is in agreement with the limited number of observations of earlier investigators. Von Tschermak (5), however, has reported the presence of lichenase in the pancreas or intestine of half the number of normal rabbits examined in connection with his studies on adaptation of enzymes to diet. Glycerol extracts of the organs were used and the fermentation test was made as a criterion of the absence or presence of a monosaccharide after incubation of the extracts of the tissue with the lichenin. Differences in technique may account for the variations between his work and that of other experimenters.

That lichenase was not uniformly distributed throughout the whole invertebrate organism was demonstrated by tests carried out on the extracts of the muscle tissue of the crayfish (*Cambarus virilis*). Repeated examination of muscle extracts of this species gave no evidence of the presence of lichenase, although extracts of the hepatopancreas of the same individuals showed marked action on lichenin. It has been suggested by von Tschermak (5) that the enzymes for the hydrolysis of lichenin and inulin are closely associated or may even be identical (inulolichenase). The inulase of *Aspergillus niger* is also able to effect the hydrolysis of lichenin (1). In the present series of experiments there is no evidence of any close association between inulase and lichenase. While lichenase was found to be present in the alimentary tract of twenty different species, inulase was observed in only one instance, in the experiments with *Lampsilis luteola*. There is no apparent relationship between the occur-

Digitized by Google

TABLE I.

Phylum.	Species.	Inulin.	Lichenin.	Raffinose.	Sucrose.	Starch.
Porifera.	1. Marine sponge (?)		+			+
Annelida.	2. Earthworm (*Helodrilus caliginosus*).	−	++	−	+	+++
"	3. Leech (*Dina sp. ?*).		+			+++
Echinodermata.	4. Starfish (*Asterias ochracea*).*	−	++		+++	
"	5. Starfish (*Asterias forbesi*).*	−	++	−	+++	+++
"	6. Sea urchin (*Strongylocentrotus drœbachiensis*).	−	++	−	+++	+++
Mollusca.	7. Black leather chiton (*Katharina tunicata*).*	−	+		−	
"	8. Giant chiton (*Cryptochiton stelleri*).*	−	++		−	
"	9. Mussel (*Unio gibbosus*).		++		++	
"	10. Mussel (*Lampsilis luteola*).	+(?)	++	+(?)	++	++
"	11. Mussel (*Alasmidonta marginata*).	−	++	−	+++	
"	12. Snail (*Planorbis trivolvis*).	−	+++	−	+++	+++
"	13. Snail (*Physa gyrina*).		++			+++
Arthropoda.	14. Crab (*Hemigrapsus nudus*).*	−	++		+++	
"	15. Crab (*Cancer productus*).*	−	+		+++	
"	16. Mud crayfish (*Upogebia sp. ?*).*	−	++		+++	
"	17. Shrimp (*Pandalus sp. ?*).*	−	++		++	
"	18. Fresh water crayfish (*Cambarus virilis*).	−	++	++	+++	+++
"	19. Grasshopper (*Melanoplus differentialis*).	−	+++	+++	+++	+++
Protochordata.	20. Sea squirt (*Cynthia sp. ?*).		+			+

* The experiments with these species were carried out during the summer of 1916 at the Puget Sound Station, Friday Harbor, Washington. Thymol was used as a preservative in these experiments instead of toluene.

Digitized by Google

TABLE II.

Vertebrates in Which the Absence of Lichenase Was Uniformly Demonstrated with Organ or Tissue Investigated.

Class.	Species.	Organ.
Pisces.	Gold fish.	Alimentary canal.
Amphibia.	Frog (adult and tadpole).	" "
Reptilia.	Horned toad.*	" "
"	Garter snake.	" "
"	Turtle (two species).	Pancreas, upper portion of small intestine.
Aves.	Domestic fowl.	" " "
Mammalia.	Rabbit.	" " "
"	Pig.	Pancreas.
"	Sheep.	Small intestine.
"	Dog.	Pancreas.
"	Man.	Saliva.

* Obtained through the courtesy of Professor A. O. Weese, of the University of New Mexico.

rence of these two enzymes in the organism of the invertebrates studied.

One other point in connection with the presence of enzymes other than lichenase is of interest. In the hepatopancreas of the two chitons (Nos. 7, 8, Table I) sucrase was absent. Since this enzyme is so widespread in its distribution examinations were made repeatedly on these species with the same negative results as far as sucrase was concerned. Of the sixteen species of invertebrates examined for this enzyme, these were the only two in which the presence of sucrase could not be demonstrated.

It was realized that with the preservative used, toluene, bacterial action was not necessarily completely inhibited. In view, however, of the uniformity of the results, positive with invertebrates, and negative with vertebrates, hydrolysis of the lichenin can hardly be ascribed to the action of the bacteria, since if bacterial enzymes are concerned there is no reason why vertebrate extracts should not be contaminated with bacteria as frequently as invertebrate extracts. Moreover, it would be difficult to understand why inulin or raffinose should not be attacked as well as lichenin since many strains of *Bacillus coli*, one of the prevailing types of organisms of the alimentary tract, ferment

Digitized by Google

raffinose and inulin readily (6). Also according to Saiki (1), *Bacillus coli* does not attack lichenin. Experiments were conducted to determine the degree of bacterial contamination in the experimental tubes in which toluene was present as a preservative. The alimentary canals of a number of grasshoppers were removed, macerated, and tests on lichenin made as before with the usual controls. After 24 hours, reducing sugar was present in considerable amounts. A bacteriological examination of the contents of the tubes was then made.[2] Agar plates showed four to eight colonies per cc. after 48 hours' incubation at 37°C. Plates from the boiled controls gave similar results. The prevailing organism was a long spore-forming bacillus of the *subtilis* group. The number of organisms can hardly have been great enough to cause the strong hydrolysis observed, especially since the unattacked control tubes showed equal bacterial contamination. Extracts of the alimentary canal of grasshoppers were also thoroughly mixed with chloroform and toluene and allowed to stand 12 hours with frequent shaking. The clear extract removed with a sterile pipette showed marked hydrolytic action on lichenin.

Chemical studies have led to few clear-cut distinctions between vertebrates and invertebrates. That a line of demarcation between the two exists in a difference in the creatine metabolism seems probable. No invertebrate tissue has been found to contain creatine, while from the muscle of the lamprey-eel, the most primitive vertebrate examined, creatine was easily isolated (7). The results of the present study on a limited number of forms suggest a second distinction between vertebrates and invertebrates in the presence of lichenase in the alimentary tract of the former. It is hoped that examination of more primitive vertebrates and higher invertebrates may show whether this difference is general or not.

[2] The bacteriological examinations were carried out by Dr. J. A. Sperry, of the Department of Bacteriology, to whom we take this opportunity of expressing our indebtedness for his cooperation.

Digitized by Google

BIBLIOGRAPHY.

1. Saiki, T., *J. Biol. Chem.*, 1906–07, ii, 251.
2. Brown, E. W., *Am. J. Physiol.*, 1898, i, 455.
3. Nilson, G., cited in *Jahresb. Thierchem.*, 1893, xxiii, 53.
4. Berg, T., *Russ. Z. Pharm.*, 1873, 129, 161; cited in *Jahresb. Chem.*, 1893, 848.
5. Von Tschermak, A., *Biochem. Z.*, 1912, xlv, 452.
6. Cf. Kligler, I. J., *J. Infect. Dis.*, 1914, xv, 187.
7. Mellanby, E., *J. Physiol.*, 1907–08, xxxvi, 447. Wilson, D. W., *J. Biol. Chem.*, 1914, xviii, 17.

Digitized by Google

Digitized by Google

THE AMOUNT AND THE DISTRIBUTION OF CREATININE AND CREATINE IN NORMAL HUMAN BLOOD.

By ANDREW HUNTER and WALTER R. CAMPBELL.

(*From the Department of Pathological Chemistry, University of Toronto.*)

(Received for publication, November 17, 1917.)

Of the non-protein constituents of blood, some, such as potassium,[1] phosphoric acid,[1, 2] lecithin,[3] free cholesterol[3, 4, 5] and amino-acids,[5, 7, 8] are localized to a greater or smaller extent within the corpuscles; some, such as sodium,[1] calcium,[1] chlorine,[1] cholesterol esters,[5] glucose,[9] and adrenalin,[10] are more abundant in, or even entirely confined to the plasma; some, finally, with urea[6, 11] as their most conspicuous known representative, maintain a practically equal concentration in both. There are few important constituents of which the relative concentration in

[1] Abderhalden, E., Lehrbuch der Physiologischen Chemie, Berlin and Vienna, 1906, 591–593.

[2] Porte, A., *Compt. rend. Soc. biol.*, 1914, lxxvii, 467.

[3] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[4] Hepner, E., *Arch. ges. Physiol.*, 1898–99, lxxiii, 595.

[5] Bloor, W. R., and Knudson, A., *J. Biol. Chem.*, 1917, xxix, 7.

[6] Bang, I., *Biochem. Z.*, 1915, lxxii, 104; 1916, lxxiv, 294.

[7] Bock, J. C., *J. Biol. Chem.*, 1917, xxix, 191.

[8] Wilson, D. W., and Adolph, E. F., *J. Biol. Chem.*, 1917, xxix, 405.

[9] Macleod, J. J. R., Diabetes; Its Pathological Physiology, London, 1913, 31. The distribution of glucose in the blood is, however, still the subject of debate. Quite recently Gradwohl and Blaivas (*J. Lab. and Clin. Med.*, 1916–17, ii, 416), using the method of Lewis and Benedict, found the sugar content of the corpuscles in man to be nearly always the same as that of the plasma.

[10] Stewart, G. N., and Rogoff, J. M., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 79.

[11] Marshall, E. K., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53. Karr, W. G., and Lewis, H. B., *J. Am. Chem. Soc.*, 1916, xxxviii, 1615. In fish blood urea is chiefly localized in the corpuscles; see Wilson and Adolph.[8]

Digitized by Google

corpuscles and plasma is unknown; but to this category belonged until quite recently creatinine and creatine.

The distribution of materials within the blood is not only a matter of considerable interest in itself; it possesses an obvious bearing upon many questions of much wider import. In the transport of materials to and from the tissues every element of the blood may play a part; but their actual passage into or out of the circulation can be effected only through the plasma. Processes like absorption, or excretion, or placental transmission may therefore raise problems for the solution of which a knowledge of concentrations in the blood as a whole is insufficient, and the determination of plasma concentrations, which may be very different, becomes a prime necessity. Creatinine and creatine, in what is already known of their behavior under physiological and pathological conditions, present a number of problems of this description.

For instance, although creatinine is being constantly excreted by the kidney, the laws which regulate its passage through that organ are unknown; any attempt to formulate them presupposes an acquaintance with the variations of the plasma creatinine, which may or may not parallel those of the creatinine of the entire blood. Creatine, on the other hand, in spite of the fact that its concentration in the blood is greater than that of creatinine, is usually absent from the urine of the male adult; but we do not know whether this is because it is localized exclusively in the corpuscles, or because its elimination, like that of glucose and chlorides, is dependent upon the crossing of a certain threshold of concentration in the plasma. The doubt could be resolved, and fresh light might be thrown upon the occasional occurrence of creatinuria, if we had precise information regarding the actual distribution of creatine in the blood. During pregnancy, to take a problem from another field, creatinine and creatine are found in the fetal as well as the maternal blood; and it is natural to inquire to what extent and in what manner these substances share in the transfer of materials from one circulation to another across the placenta. Obviously the question demands for its answer a knowledge of their concentrations in the respective plasmas. Even in pathological conditions a study of the relations under discussion might well reveal something of interest. The accu-

Digitized by Google

mulation of creatinine in the blood has shown itself to be a useful index of renal insufficiency; but we are as yet unaware whether the excess present in the circulation of a nephritic permeates all the elements of the blood, or accumulates in the plasma alone. If the latter alternative should prove to be correct, the variations of the plasma creatinine in kidney disease would be even more striking than those of the whole blood creatinine, and would form a still more delicate index of the organ's capacity to excrete. A separate study of plasma and whole blood creatinine and creatine in different pathological conditions might even reveal significant variations in the permeability of the corpuscles for these substances.

It was with these considerations in mind that we undertook recently to add creatinine and creatine to the list of blood constituents with more or less definitely established distribution.

In a preliminary communication,[12] in which each aspect of our interest in the problem was distinctly indicated, we have already presented an abstract of our earliest results. This constituted, as far as we have been able to discover, the first published contribution to the subject in relation to human, or indeed mammalian blood; although some data upon the distribution of creatinine and creatine in fish blood were simultaneously communicated by Wilson and Adolph.[8] Among the conclusions which we thought to be justified by our observations the chief was that both in creatinine and in creatine the corpuscles are under nearly all conditions richer than the plasma. So long as the reliability of our methods was taken for granted no other conclusion was possible. A continuation of the work along the original lines did nothing to modify the general tendency of our analytical results, but it gradually engendered doubts concerning their real significance. These doubts were not a little accentuated by the work of Wilson and Plass,[13] who, dealing a little later with the problems upon which we were engaged, confirmed in a general way our view regarding the distribution in human blood of creatine, but upon that of creatinine reached a different

[12] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1917, xxix, p. xviii.

[13] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

Digitized by Google

conclusion. We therefore deemed it necessary, before publishing a fuller account of our work, to satisfy ourselves upon the precise value of the analytical methods which we had employed. The issue of this incidental, but necessary inquiry, the details of which have been recorded in another paper,[14] was such as to necessitate a revision of some of our original conclusions. During its progress we confined our attention, in the accumulation of additional data, almost entirely to the fundamental points of the amount and the distribution of creatinine and creatine in normal human blood. It is to the results bearing upon these points, and to the interpretation of them which now appears to us the most plausible, that the present paper is principally devoted. Other problems, such as the relation of blood creatine to creatinuria, are touched upon only in so far as incidental observations offer occasion. It is hoped that the opportunity may yet arise to carry the inquiry further along each of the lines we have proposed.

Subjects.

The blood specimens, which for our present purpose were assumed to be normal, numbered 60, and were derived from 56 individuals, who fall into three main groups. The first, Group I, consists of 26 male students and instructors, all, as far as could be judged, in perfect health; 2 of these provided three specimens each of blood so that the total number of analyses performed within this group was 30. The second, Group II, includes 20 hospital patients, male and female, mostly convalescent after childbirth or purely surgical complaints, and all free, as far as known, from organic disease. This group is divided into two subgroups; but the distinction between them is one of date only, Group II A having been studied several months earlier than II B. The final group, Group III, is formed by 10 healthy women in a late stage of pregnancy, of whom 1 only was as yet confined to bed.

No subject has been admitted to any of these groups in whom a routine examination revealed evidence of organic disease. In this respect all equally were normal individuals; but of course

[14] Hunter and Campbell, *J. Biol. Chem.*, 1917, xxxii, 195.

Digitized by Google

the figures yielded by Group I possess a rather better claim than the others to be regarded as standards. The patients of Group II were not exactly in the best physical condition, nor were they living under altogether normal circumstances; while, with reference to Group III, we cannot at present be certain that even a normal pregnancy is entirely without effect upon the relations we are studying. The evidence furnished by Groups II and III is therefore to be taken as suggestive or confirmatory rather than conclusive in itself.

Methods.

The blood was taken from a superficial arm vein by means of a syringe, and its coagulation was prevented by the prompt addition of 20 per cent potassium oxalate in as nearly as possible the proportions prescribed by Folin.[15] The plasma was immediately separated from a portion by centrifugation. While this was being done, the subject was usually required to provide a specimen of urine. The analyses planned were then carried out with the briefest possible delay. They included the determination of total and preformed creatinine in whole blood, plasma, and urine, together with the estimation, by means of the simplified hematocrit of Epstein,[16] of the relative plasma volume in the oxalated blood.[17] For the creatinine and creatine determinations in blood and plasma we employed the methods of Folin,[15] although the technique followed was not always precisely the same. Thus in many of our earlier observations we followed the directions of Myers and Fine[18] in laking the blood before saturating it with picric acid; and in our creatine determinations we did not take the precaution, which we have lately[14] found to be desirable, of so diluting blood and plasma in every case that the same standard could be employed throughout the series. In one respect at

[15] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[16] Epstein, A. A., *J. Lab. and Clin. Med.*, 1915–16, i, 610.

[17] In many cases, but not all, we had sufficient blood to determine also the total non-protein nitrogen. It seemed hardly worth while to report individually the scattered results thus obtained. It may be stated that they ranged from 29 to 40 mg. per 100 cc., and that, as far as they went, they revealed no constant relation to the data for creatinine.

[18] Myers, V. C., and Fine, M. S., Chemical Composition of the Blood in Health and Disease, Cooperstown, N. Y., 1915, 19.

Digitized by Google

least our practice was absolutely uniform; the creatinine value of each color reaction was obtained by reference to the standard curves which we have recently described.[19] For the determination of creatinine and creatine in urine we usually employed the macro method, in which case the conversion of creatine was accomplished by the procedure of Benedict.[20] In a few instances we utilized the micro method of Folin.[21] The chief purpose of the urine analysis was the detection and measurement of the creatine. The creatinine results, which have yielded little of value in themselves, are recorded chiefly for the sake of completeness.

The urine specimens were subjected also to a routine qualitative examination.

Results.

The analytical results are shown in Table I.[22] They are divided into the groups and subgroups already mentioned, and are arranged within each group so as to present an ascending scale of plasma creatinine. Certain other ways of arranging the results illustrative of special points discussed in the text are employed in the general averages at the end of the table. The corrections introduced into the table are made, as explained later, on the basis of conclusions reached in our critical examination of the methods.[14] The few figures given for creatine of the corpuscles are calculated from the whole blood and plasma creatine in conjunction with the relative plasma volume.

DISCUSSION OF RESULTS.

It has been shown[14] that of the four creatinine determinations required in the complete analysis of blood that of the preformed creatinine of plasma has the best claim to accuracy. It is for this reason that we selected the plasma creatinine to determine the arrangement of our results; and for the same reason we shall discuss its variations before attempting to estimate the significance of our other data.

[19] Hunter and Campbell, *J. Biol. Chem.*, 1916–17, xxviii, 335.

[20] Benedict, S. R., *J. Biol. Chem.*, 1914, xviii, 191.

[21] Folin, *J. Biol. Chem.*, 1914, xvii, 469.

[22] Many of them are taken from the thesis submitted by W. R. Campbell in partial fulfillment of the requirements for the degree of M.D., University of Toronto, 1917.

Digitized by Google

Plasma Creatinine.

In our preliminary communication[12] we stated that the plasma creatinine of normal individuals is always less than 1.0 mg. per 100 cc. This opinion was based on the nine results which constitute Group II A of the larger series here presented. It is now apparent that these results were by no means completely representative. The figures of Group I show that at least for healthy and active males the ordinary level of the plasma creatinine is somewhat higher than our early estimates. Of the 30 results within that group no more than 8 are below 1.0 mg. A majority (17) are included by the values 1.1 and 1.2. The lowest is 0.80, the highest 1.3. The average of the whole group is 1.09, or in round figures 1.1. These data are furnished by a number of separate individuals (26) sufficiently large to make them fairly representative of their class.

When it was found that Group I, our second series in point of time, gave generally higher values than Group II A, our first, we were inclined for a little to question the correctness of the earlier analyses. We therefore, by way of control, supplemented these at a considerably later date by a further series of eleven determinations upon strictly comparable material. The results, constituting Group II B, do not differ in any essential respect from those of Group II A. One, it is true, shows a value as high as any found even in Group I; but of the remainder none exceeds 1.0 mg., and eight are below it. The results in the two subgroups therefore mutually confirm one another, and form together a sufficiently concordant single series. Taking them as such we find that the plasma creatinine of Group II ranges from 0.70[23] to 1.3, or, neglecting the last as a distinctly aberrant value, to 1.0. The average of all is 0.89, or, if we again exclude

[23] We have in our records one case only where the plasma creatinine appeared to be lower even than the minimum here reported. It was that of a female patient without known organic disease, supposed to be suffering from hysteria, who might fairly enough have been included in Group II. Her plasma creatinine was 0.58. This value remained so unique that, lacking opportunity to confirm it, we have hesitated to place confidence in the correctness of the analysis, and have therefore omitted it from the table.

Digitized by Google

the maximum from the calculation, 0.86. It is clear that the hospital inmates selected as normal individuals presented in general an unmistakably lower level of plasma creatinine than the active male students.

With respect to the cause or causes of this difference we can only speculate. Group II, unlike Group I, included a considerable proportion of females. Several of its members showed an abnormally high relative plasma volume, possibly indicative of a certain degree of dilution of the blood. Three-fourths of them were confined to bed, and the others were at least leading a sedentary existence. All, it is safe to assume, were eating less than the more actively employed members of Group I, and some, it is known, were on a very restricted diet. It is not yet possible to decide what actual influence any or all of these circumstances may have exerted upon the plasma creatinine of the group as a whole. Certainly neither the first mentioned nor the second will wholly explain its relative lowness. For, while it is true that the average for the females of the group is lower than that for the males, and even that within each subgroup the maximum for women is lower than the minimum for men, yet the males, considered apart, still yield distinctly less creatinine than those of Group I; and, although dilution of the plasma doubtless may diminish its creatinine concentration, there is evidence on the one hand (Cases 58, 59, 60) that it does not always do so, and on the other (Cases 3, 5, 36) that a plasma creatinine below the average may be encountered in blood which is the reverse of hydremic. Of the other two possible factors suggested, low diet and enforced inactivity, the latter is the one which seems the more likely to be of general importance; for the production of creatinine within the body is independent of the diet but intimately related to the condition of the muscular tissues.[24] It may well be assumed that few, if any, of our hospital subjects maintained themselves, while confined to bed, upon the same plane of muscular efficiency as the members of Group I. Provisionally therefore we are inclined to ascribe the low plasma creatinines of Group II partly to the female sex of some of its members, but largely to lack of exercise in its effect upon mus-

[24] Shaffer, P. A., *Am. J. Physiol.*, 1908–09, xxiii, 1.

Digitized by Google

cular tone. If the associations thus suggested are correct, a low plasma creatinine would be characteristic of such cases as might be expected to have a low creatinine coefficient; and it is of some interest that according to Shaffer[25] there is found under similar conditions a smaller concentration than usual of creatinine in the muscles also. The possible influence of prolonged inaction upon the plasma creatinine could obviously be put to experimental proof; and a further examination of the question is accordingly in contemplation. Meanwhile it may be of practical clinical importance to note that a plasma creatinine of 1.2 mg., which would be perfectly normal in an active subject, already verges upon the pathological for one who is resting in bed.

The idea, suggested by the results within Group II, that females have naturally a lower plasma creatinine than males, receives additional support from the data furnished by the subjects of Group III. These, as already stated, were all females, in whom pregnancy constituted the only deviation from normality. The ten plasmas included in this group contained amounts of creatinine ranging from 0.71 to 1.2, seven of them falling below 1.0 mg., and the average of all reaching only 0.92. These figures certainly occupy a lower general level than those of Group I, with their minimum of 0.80 and their average of 1.1. It may, of course, be objected, that women advanced in pregnancy, even if they are still taking a certain amount of exercise, can hardly be regarded as physiologically comparable with active young men. The comparison, at the end of Table I, of the general average for all males (1.04) with that for all females (0.87) has to be regarded with similar reserve. It is really only within Group II that difference of sex is not obviously complicated by some other circumstance of possible consequence. We are therefore not yet prepared to state with conviction, although we regard it as exceedingly probable, that the sex effect indicated by our figures has an existence independent of all other factors. To determine finally its actual importance, it would be necessary to possess for comparison with the data of Group I a series of observations upon active and healthy young women; unfortu-

[25] Shaffer, *J. Biol. Chem.*, 1914, xviii, 525.

Digitized by Google

nately the opportunity of collecting such a series has not yet arisen.[26]

There is no evidence in any of the groups that the variations of the plasma creatinine are definitely related to the age of the subject, at any rate within the limits of 12 to 55 years.

Creatinine of Whole Blood and Its Distribution.

In each of the 60 pairs of preformed creatinine determinations included in the table the whole blood yielded a higher value than the plasma. As far, therefore, as the immediate outcome of the analysis is concerned, our original report[12] receives here the fullest corroboration. If the colorimetric method were of the same accuracy in blood as in plasma, it would follow at once that, as we formerly announced, the creatinine of the blood is chiefly concentrated in the corpuscles. Unfortunately the condition of equal accuracy is not fulfilled. We have recently,[14] on a careful examination of the method, convinced ourselves that, as Wilson and Plass[13] had already assumed, the values reported for whole blood are an exaggeration of the truth. The extent of the exaggeration we found to vary with the individual instance and the particular technique employed. With the original technique of Folin,[15] modified only by the use of standard curves[19] in the interpretation of the colorimeter readings, the error, as we estimated it, amounts on the average to about 50 per cent of the quantity actually present. While therefore it is not possible to apply to each of the single determinations in our table a uniform correction, we are in a position to correct the average of any group of determinations in which the blood was not laked. Two such averages appear in Table I; one is that of 17 out of the 30 results in Group I, the other that of 27 (including the above 17) out of the total 60. The two averages are practically identical (1.57 and 1.58). If these are 50 per cent in excess of the true average, the latter will be found by deducting from each one-third. This gives a corrected average of 1.05 for both cases.

[26] Plass (*Bull. Johns Hopkins Hosp.*, 1917, xxviii, 137) states that the serum creatinine of women in parturition is the same as that of normal non-pregnant women, which again is somewhat lower than in normal men; but detailed data in support of these statements are not presented.

Digitized by Google

A comparison of this corrected average with the averages for the two corresponding groups of plasmas (1.09 and 1.03) shows that the difference in the creatinine content of whole blood and plasma is in reality practically negligible. It is, of course, not possible to say that there may not be in individual specimens a slight excess of creatinine in corpuscles or in plasma; but the conclusion to which the twenty-seven results considered indubitably appear to point is that in general the *creatinine of normal human blood is distributed among its different elements at a practically uniform concentration.* Our previously expressed opinion upon the point is therefore abandoned, and the contention of Wilson and Plass sustained.

Lacking quantitative data upon the average error of the Myers' technique, we are unable to apply any appropriate correction to results obtained upon blood which had been laked. Those figures, therefore, which are in parentheses in the table, cannot be utilized in the present argument. It will be noticed that the disproportion between them and the corresponding plasma results is in general greater than in the case of unlaked blood. Thus, the average for all 60 bloods, including as it does 33 which were laked, is not 50 but 75 per cent higher than the plasma average; and in individual cases, especially, it would seem, among puerperal women, laked blood appears to have twice or even thrice as much creatinine as its plasma. All this is in accord with our previous observations, and those of Wilson and Plass, upon the excessive tendency of the Myers' procedure to exaggerate the whole blood creatinine. It may be remarked, though, that this tendency is much less obvious in the strictly normal cases of Group I than in either of the other groups.

The conclusion that the creatinine content of blood is in reality the same as that of its plasma has an obvious bearing upon the question of the normal limits within which the whole blood creatinine may range. According to Folin and Denis[27] these are 1.0 to 1.4 mg. per 100 cc.; according to Myers and Fine,[28] 1.0 to 2.0. With such estimates as these our own uncorrected results with whole blood are in substantial agreement.

[27] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 487.

[28] Myers and Fine, *Post-Graduate*, 1915, xxx, 39.

Digitized by Google

Our minimum is 1.1, our maximum 2.8; if results in puerperal women, which appear to be aberrant, are excluded, our maximum is reduced to 2.4, or, if we further limit the record to analyses of unlaked blood, to 2.1. Now, if it is granted that creatinine is uniformly distributed throughout the blood, the correct creatinine content of the latter will be given not by the figures just quoted, which we believe to exceed the truth, but by those for plasma, which we have shown[14] to be substantially free from error. On that basis the normal range of blood creatinine is found to be 0.70 to 1.3, with an average of practically 1 mg. This range is all but identical with that which may be found among results for human plasma reported by Plass[26] and by Wilson and Plass.[13] It indicates for the true creatinine content of blood a somewhat lower general level than the figures of Myers and Fine or even of Folin and Denis. It remains, nevertheless, of the same order of magnitude as these, and is very far from approaching the extraordinarily low estimate of Gettler and Baker.[29] These authors found that out of 30 bloods 26 contained only 0.1 mg. or less of creatinine per 100 cc.; and Gettler,[30] in a subsequent analysis of 11 other specimens, indicates 0.5 as the upper limit of normality, and reports only 5 as having more than 0.1. For such results as these we have failed to find either confirmation or explanation. The work of Gettler may be admitted to prove (see his Experiment V[30]) that the limits of 1.0 to 1.4, as found by Folin and Denis, should be corrected to 0.95 to 1.7; but that will hardly serve to explain how Gettler and Baker obtain values as low as 0.1.

Relation between Plasma and Urinary Creatinine.

The determination of urinary creatinine was undertaken simply as a step in the detection and estimation of creatine, and not with any deliberate intention of studying the laws regulating its own passage through the kidney. Any such purpose would have necessitated the taking of many precautions which for the present were neglected. It did, nevertheless, seem possible that even

[29] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.
[30] Gettler, A. O., *J. Biol. Chem.*, 1917, xxix, 47.

Digitized by Google

the haphazard procedure followed might reveal some rough relationship between the plasma and the urinary concentrations of creatinine. As far as comparison of the two in individual cases goes, no indication whatever of any such relationship can be detected. On the other hand, when regard is paid only to the various averages scattered through Table I, a certain degree of correspondence becomes apparent. Indeed, if one limits the attention to the averages in which the number of urines concerned is more than ten, the correspondence becomes a fairly close one. The averages in question are for plasma 0.87, 0.89, 0.99, 1.03, 1.04, 1.09, and for urine 95, 124, 141, 166, 157, 163. It seems probable, therefore, that the plasma concentration is at least one among the many factors upon which the concentration of creatinine in the urine must depend.

The Distribution of Creatine in Blood.

The greater number of our creatine determinations had been already carried out, before it became obvious, through the work of Wilson and Plass,[13] Greenwald,[31] and ourselves,[14] that the method employed, which in the beginning was the only one available, fell more than a little short of the accuracy necessary to our purpose. It seemed then best, instead of turning to other methods of as yet unproved reliability, to complete the series as it had been commenced, and to hope that, when due allowance had been made for the errors incurred, the results might prove to be not altogether without significance. The hope has been, we think, to a certain extent realized. At the same time we cannot, for conclusions based upon admittedly inaccurate data, claim more than a provisional validity. In the discussion which follows we wish therefore to be understood as dealing for the most part with suggestion rather than proof. Many, if not all, of the deductions drawn from our figures demand confirmation by a less dubious analytical method.

On one point perhaps the results do actually amount to a demonstration. In every case the plasma yields for creatine a figure so far below that of the blood, that there can hardly exist a doubt that the bulk of that substance is carried in the cor-

[31] Greenwald, I., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 115.

Digitized by Google

puscles. This conclusion, already formulated in our earlier contribution[12] to the subject, and later confirmed by Wilson and Plass,[13] is not affected by the respective errors of the creatine determinations in blood and in plasma; for these are of such relative magnitude that existing differences are more likely to be concealed by them than exaggerated. We have shown[14] reason to suspect that the true creatine content of the blood is, roughly speaking, about one-half, and that of the plasma about one-fourth, of the amount indicated in the colorimetric analysis. This estimate is not of such a precise character that it can be fairly applied to individual pairs of results, but it is perhaps not too crude to be used in a tentative correction of our averages. Modified accordingly, these averages vary, in the different groups distinguished, from 2.7 to 3.5 in the case of whole blood, and from 0.40 to 0.62 in the case of plasma. The corrected averages for all cases in which creatine was determined at all are 2.97 (practically 3.0) and 0.46. In general the proportion of whole blood to plasma creatine is about 6 or 7 to 1. Taking the average values reached along with the average plasma volumes corresponding, it is possible to calculate the average amount of creatine in the corpuscles; this appears to lie, in one group or another, between 6 and 9, with an average for all cases of 6.7 mg. per 100 cc.[32] It may be remarked that all the figures for creatine, and especially those for corpuscular creatine, are higher in the female groups than in the male. These quantitative data are offered, as we have said, with due reserve and as first approximations at the best.

[32] There are no available data with which our estimates of corpuscular creatine can be directly compared; but Plass (*Bull. Johns Hopkins Hosp.*, 1917, xxviii, 297), who employs an improved analytical method, has recently published figures ranging from 5.99 to 15.29 (average, 10.8) as the *total* corpuscular creatinine of ten parturient women. He states further, without recording details and without indicating clearly whether he is dealing here with creatine or total creatinine, that non-pregnant women yield much lower values (6.2 to 6.5), and that in one male the corpuscular concentration was lower still (4.9). Our figures, it will be seen, agree with those of Plass in indicating a smaller proportion of creatine in the corpuscles of the male, and a special accumulation in those of pregnant women. Even with regard to the absolute amounts present there exists a gratifying measure of agreement between results obtained in such different ways.

Digitized by Google

If they should be confirmed, it would follow, after what has been said of the equal distribution of creatinine, that the corpuscles contain from 5.5 to 10 times as much creatine as creatinine,[33] while in the plasma it is the latter which is predominant.

Relation between Creatine of Plasma and of Urine.

This leads us to consider whether our results throw any light upon the question of the relation between the concentration of creatine in the plasma and its occasional appearance in the urine. Assuming that the permeability of the healthy kidney cells to creatine is constant, there would appear to be only two forms which this relation might assume; either creatine is promptly excreted upon its earliest appearance in a plasma which is normally creatine-free, or else it passes the kidney only when its plasma concentration rises above a certain definite threshold.[34] Now, if the individual data for plasma and urinary creatine are compared, it is impossible to detect any uniform relation between them. Plasma creatines of from 1.4 to 3.2 seem to be associated indifferently with the presence of creatine in the urine or its absence. It can perhaps be said that whenever the (uncorrected) plasma creatine is lower than 1.4 the urine is creatine-free, and that whenever it is above 3.2 creatinuria makes its appearance; but the generalization includes in its scope such a small proportion of our cases that it possesses little significance. This apparent lack of a consistent relation is probably in part the consequence of the variable error involved in the single determination of plasma creatine. At any rate, when we compare averages, in which the error may be supposed to be more nearly constant, a certain regularity becomes at once apparent.

In order to exhibit this more clearly we have in the

[33] In our preliminary communication[12] (p. xix), in the sentence dealing with the relative amounts of creatinine and creatine in the corpuscles, the words "first" and "second" are transposed in such a manner as to convey a meaning exactly the opposite of what was intended. When the abstract was printed this unfortunately escaped detection.

[34] The dependence of creatinuria upon a specific increase of renal permeability constitutes a third possibility which cannot be entirely excluded, but for which there would not exist an exact analogy.

Digitized by Google

TABLE I.

No.	Case.	Age.	Sex.	Blood.					Urine.		Remarks.
				Creatinine.		Creatine as creatinine.		Relative plasma volume.	Creatinine.	Creatine as creatinine.	
				Whole blood.	Plasma.	Whole blood.	Plasma.				
						Group I.					
				mg.	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	
1	Y. B.....	25	M.	(1.8)	0.80	5.2	1.3	54	144	0	
2	P. R. S.....	31	"	1.2	0.84	6.8	2.0	60	95	5	
3	J. M.....	26	"	(1.5)	0.92	6.1	3.2	48	121	0	
4	A. B. H.....	24	"	1.6	0.92	5.7	1.9	63	182	7	
5	E. C. T.....	22	"	(1.9)	0.92	5.6	1.3	50	97	0	
6	C. A. Wa.....	25	"	1.4	0.93	5.3	1.9	61	61	2	
7	F. R. S.....	23	"	1.3	0.96	5.7	1.4	60	162	0	
8	R. C. C.....	24	"	1.3	0.97	5.8	1.3	58	259	0	
9	J. H. H.....	24	"	1.3	1.0	5.8	1.3	60	308	0	
10	G. C. M.....	30	"	1.6	1.0	6.0	1.4	61	60	3	
11	H. A. R.....	25	"	(1.8)	1.0	5.3	1.8	58	208	37	
12	R. C. M.....	23	"	(1.7)	1.1	4.2	1.3	54	97	0	
13	C. A. We.....	27	"	2.1	1.1	4.4	1.4	57	104	7	
14	R. H.....	24	"	(1.8)	1.1	7.0	2.2	59	231	0	
15	A. G.....	25	"	1.6	1.1	5.8	1.6	63	210	0	
16	A. H.....	40	"	(1.5)	1.1	4.7	1.4	62	88	0	
17	W. R. C.....	26	"	1.4	1.1	5.3	1.8	63	—	—	11 weeks later than Nos. 18 and 19.
18	"			(1.5)	1.1	4.3	1.1	63	144	0	
19	"			(1.6)	1.2	4.4	1.4	57			Same blood as No. 18, but hirudinized instead of oxalated.
20	A. H.....	40	"	(1.6)	1.2	4.6	1.6	57			Same blood as No. 16, but hirudinized instead of oxalated.

Digitized by Google

21	H. K. D.	30	M.	1.8	1.2	5.5	1.6	57	308	0	
22	D. S. M.	30	"	1.9	1.2	5.7	1.2	59	140	0	
23	F. M.	33	"	1.8	1.2	5.6	1.4	58	164	0	
24	C. O. M.	27	"	(1.5)	1.2	6.5	2.3	57	107	0	
25	W. A. S.	32	"	(1.7)	1.2	5.3	2.5	56	95	0	
26	C. V. S.	26	"	1.5	1.2	5.1	1.3	62	180	0	
27	T. S.	27	"	(2.1)	1.2	7.3	2.3	58	172	0	
28	J. R. S.	27	"	1.7	1.2	5.3	1.1	64	216	0	
29	J. Re.	28	"	1.6	1.3	5.9	2.9	61	274	20	
30	A. H.	40	"	1.8	1.3	—	—	61	—	—	13 weeks later than Nos. 16 and 20.
Average of Group I.				(1.63)	1.09	5.52	1.70	59	163	3.1	
Same, corrected.				—	1.09	2.76	0.42				Creatine of corpuscles = 6.1.
Average of laked bloods (13).				(1.69)	1.08	5.42	1.82	57			
" " unlaked " (17).				1.58	1.09	5.61	1.59	61			
Same, corrected.				1.05	1.09	2.80	0.40				Creatine of corpuscles = 6.6.
Group II A.											
31	G. H.	23	F.	(1.2)	0.72	6.1	2.0	61	124	14	Chronic constipation. In bed.
32	G. D.	55	M.	(1.8)	0.76	4.8	1.0	54	74	0	Sprain. In bed.
33	W. D.	35	"	(2.2)	0.80	6.9	1.7	63	132	0	Hernia, 10 days after operation. In bed.
34	W. Be.	29	"	(1.8)	0.81	3.7	1.3	64	101	0	Undescended testicle, 7 days after operation. In bed.
35	W. T.	48	"	(2.2)	0.83	5.2	1.6	59	46	11	Hydrocele, before operation. Ambulant.
36	J. C.	26	"	(1.8)	0.91	6.9	1.0	46	247	0	Fracture. Ambulant.
37	E. F.	21	"	(1.4)	0.91	7.4	1.3	54	84	2	Hernia, before operation. In bed.
38	P. V.	12	"	(1.7)	0.92	4.5	1.4	66	129	8	Lacerated thumb. In bed.
39	G. M.	25	"	(2.0)	0.98	4.2	1.4	55	152	4	Hernia, 7 days after operation. In bed.

* Determinations for the figures in parentheses were obtained by Myers' technique. Averages in which these were included are likewise in parentheses.

Digitized by Google

TABLE I—*Concluded.*

No.	Case.	Age.	Sex.	Blood.					Urine.		Remarks.
				Creatinine.		Creatine as creatinine.		Relative plasma volume.	Creatinine.	Creatine as creatinine.	
				Whole blood.*	Plasma.	Whole blood.	Plasma.				
				Group II B.							
				mg.	*mg.*	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	
40	V. S.	18	F.	(1.7)	0.70	9.5	3.5	69	125	65	Puerperium, 9th day.
41	F. K.	23	"	1.5	0.78	7.2	1.9	67	53	5	Hysteria. In bed.
42	L. Le.	22	"	(2.6)	0.80	5.1	1.9	59	79	28	Puerperium, 8th day.
43	M. W.	35	"	1.5	0.86	7.3	2.1	67	43	12	Hysteria. In bed.
44	E. H.	19	"	(2.8)	0.87	4.8	2.0	57	99	39	Puerperium, 11th day.
45	E. T.	19	"	(2.3)	0.91	5.7	3.6	60	165	57	" 8th "
46	J. R. C.	39	M.	1.8	0.92	—	—	67	—	—	Nerve suture. In bed.
47	A. C.	40	"	1.5	0.95	6.8	2.1	61	—	—	Fracture. In bed.
48	J. B.	28	"	1.6	1.0	6.3	2.0	60	178	0	" Ambulant.
49	W. Br.	29	"	1.5	1.0	6.0	1.4	62	277	0	" "
50	F. D.	32	"	2.0	1.3	—	—	63	—	—	After hernia operation. Ambulant(?).
Average of Group II				(1.85)	0.89	6.02	1.84	61	124	14.4	
" " " " corrected				—	0.89	3.01	0.46				Creatine of corpuscles = 7.0.
" " males only				(1.79)	0.93	5.70	1.47	60	142	2.5	
" " females "				(1.94)	0.81	6.53	2.43	63	98	31.4	

Digitized by Google

Group III.

51	J. E.	36	F.	1.3	0.71	7.7	—	—	—	—	Ante partum, 8 days.
52	J. Ri.	18	"	(1.7)	0.72	5.5	1.8	61	95	17	" " 5 weeks.
53	B. S.	29	"	(1.7)	0.78	6.6	1.9	72	100	0	" " 19 days.
54	C. G.	28	"	(2.0)	0.82	5.5	2.4	59	67	—	" " 4 "
55	F. W.	37	"	1.8	0.89	7.9	—	—	96	34	" " 4 "
56	L. Li.	32	"	1.1	0.89	6.9	—	—	111	10	" " 2 "
57	B. C.	23	"	(1.7)	0.99	6.0	2.5	64	81	27	" " 1 day.
58	B. R.	28	"	(2.0)	1.1	9.6	2.3	71	—	—	" " a few hours.
59	E. R.	29	"	(2.1)	1.1	9.5	3.4	68	—	—	" " 11 days.
60	M. F.	25	"	(2.4)	1.2	4.8	3.0	70	—	—	" " 15 "
Average of Group III				(1.78)	0.92	7.00	2.47	66	92	17.6	
" " " " corrected				—	0.92	3.50	0.62				Creatine of corpuscles = 9.1.

General averages.

All cases (60)	(1.73)	0.99	5.94	1.85	60	141	8.6	
Same, corrected	—	0.99	2.97	0.46				Creatine of corpuscles = 6.7.
All males (43)	(1.68)	1.04	5.57	1.64	59	157	2.9	
All females (17)	(1.85)	0.87	6.81	2.45	65	95	25.7	
All with blood unlaked (27)	1.57	1.03	6.08	1.67	61	166	5.0	
Same, corrected	1.05	1.03	3.04	0.42				Creatine of corpuscles = 7.1.

* Determinations for the figures in parentheses were obtained by Myers' technique. Averages in which these were included are likewise in parentheses.

Digitized by Google

first part of Table II arranged all the averages for plasma creatine (none of which is drawn from less than seven separate determinations) in ascending order of magnitude, placing beside each of them the average urinary creatine of the group to which it belongs. The figures are then seen to fall readily into three sets. In the first, consisting of four examples with a plasma creatine (corrected) of about 0.4, the urinary creatine is never greater than 5; in the second, two examples with a plasma creatine of 0.46, the urinary creatine lies between 8 and 15; while in the third, three examples with a plasma creatine of about 0.6, the urinary creatine ranges from 17 to 32. Unfortunately the evidential value of this apparent correspondence is not so great as it seems; for, in consequence of the occasional incompleteness of the analytical record, the respective averages for plasma and for urine are not invariably drawn from the same set of individuals within the group which they are taken to represent. From this objection the figures which constitute the second part of Table II are free. They are averages which have been computed from those cases only in which *both* urine and plasma were successfully analyzed for creatine. The cases conforming to this requirement (46 in number) have been regrouped with reference simply to the concentration in which creatine was being excreted at the time of the analysis. Thus in the first group (26 cases) the urine was creatine-free; in the second (9 cases) it contained less than 10 mg. per 100 cc.; the third included all of the 20 cases in which any creatine at all was present; while the fourth embraced those only (11) in which the concentration of creatine was higher than 10 mg. per 100 cc. The data yielded by these groups indicate with clearness the association of a regular increase in the average creatine content of the urine with an equally regular, though of course much more gradual rise in the average level of the plasma creatine. They therefore afford an unequivocal confirmation of the conclusions to which the first part of the table had already pointed,—that the concentration of creatine in the urine bears a direct relation to that in the plasma, and that a slight increase in the latter (as little as 0.1 mg. per 100 cc.) suffices to bring about a copious excretion.

Had it been possible to put absolute confidence in the analytical data for plasma creatine, the last results would have demon-

Digitized by Google

TABLE II.

Comparison of Average Creatine Contents of Urine and Plasma.

Description of group.	No. of cases.	Plasma creatine per 100 cc.		Urinary creatine per 100 cc.
		Found.	Corrected.	
A. Grouping determined by case classification.				
		mg.	*mg.*	*mg.*
Males of Group II	11	1.47	0.37	2.5
All males	40	1.64	0.41	2.9
" with blood unlaked	21	1.67	0.42	5.0
Group I (males)	29	1.70	0.42	3.1
" II (males and females)	18	1.84	0.46	14.4
All cases	54	1.85	0.46	8.6
Females of Group II	7	2.43	0.61	31.4
All females	14	2.45	0.61	25.7
Group III (females)	7	2.47	0.61	17.6
B. Grouping determined by concentration of urinary creatine.				
Urines creatine-free	26	1.59	0.40	0
" containing less than 10 mg	9	1.62	0.41	4.8
" " any creatine whatever	20	2.02	0.51	18.5
" " more than 10 mg	11	2.34	0.59	29.7

strated one point more; the existence, namely, of a threshold for creatine, the level of which they would have fixed sharply at 0.4 mg. per 100 cc. But the unreliability of the determination renders exceedingly doubtful the significance of a quantity as small as that. Indeed the extreme lowness of the threshold indicated (it would be surprisingly low even if the original figures needed no correction) is enough in itself to throw suspicion upon its reality. Thresholds of established importance, like those of glucose and chlorides, seem to be generally of much greater magnitude. The idea that there may be a normal constant of this sort for creatine receives moreover, it must be admitted, no support from the results of Wilson and Plass,[13] who used a method of analysis presumably more accurate than ours. The figures they present indicate rather the complete absence of creatine from the plasma in cases where the urine is ordinarily creatine-free, and its presence in those only where creatinuria

Digitized by Google

is the rule. Their evidence, unfortunately, is in some respects as defective as our own. The number of human cases they report is but nine in all, and in none of them was the urine actually analyzed. A final settlement of the question of a creatine threshold must therefore await the collection, by methods of unquestioned accuracy, of data more complete and reliable than any that we yet possess.

SUMMARY.

1. The creatinine content of normal human blood plasma ranges under different conditions from 0.70 to 1.3 mg. per 100 cc., the average for 60 specimens examined being 1 mg.

2. It is practically certain that the creatinine of normal blood is distributed through corpuscles and plasma in uniform concentration.

3. The figures given for plasma indicate, therefore, also the true creatinine content of whole blood.

4. The blood creatinine is apt to be lower in females than in males, and lower in subjects deprived of exercise than in those leading an active life. It is suggested that the blood creatinine is related to muscular development in much the same way as the creatinine coefficient.

5. The creatine of the blood is chiefly concentrated in the corpuscles. With the method used exact determinations were unattainable, but it is roughly estimated that the average creatine content of the corpuscles lies between 6 and 9 mg. per 100 cc., while that of the plasma is not more than 0.4 to 0.6. The blood as a whole contains apparently an average of about 3 mg. per 100 cc. There seems to be more in the blood of females than of males.

6. There is a distinct correspondence between increase of plasma creatine and the appearance of creatine in the urine; but whether the plasma, in the absence of creatinuria, is creatine-free or whether there exists a threshold for creatine excretion, has not been positively determined. If there is a threshold, it is a very low one.

We take this opportunity of thanking the students and instructors of Toronto University Medical School who permitted us to

Digitized by Google

examine specimens of their blood. It is a pleasure also to acknowledge the courtesy with which Doctors B. P. Watson, K. C. McIlraith, A. McPhedran, G. W. Ross, W. B. Thistle, F. N. G. Starr, and G. A. Bingham, all of the staff of the Toronto General Hospital, permitted us to use material from the wards under their charge.

Digitized by Google

Digitized by Google

THE INFLUENCE OF THYROID FEEDING UPON CARBOHYDRATE METABOLISM. I.

THE STORAGE AND MOBILIZATION OF THE LIVER GLYCOGEN IN THYROID-FED ANIMALS.

By SHIGENOBU KURIYAMA.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, November 24, 1917.)

An earlier paper[1] dealt chiefly with the influence of thyroid feeding upon the glycogen content of the liver and the sugar content of the blood. In the present series, I have investigated the glycogenetic and glycogenolytic action of the liver of thyroid-fed animals, the influence of thyroid feeding upon the liver glycogen of frogs, the interrelations of the thyroid and adrenals, acidosis after thyroid feeding, and the influence of alkali upon the diminished content of liver glycogen of thyroid-fed animals. Desiccated thyroid (Parke, Davis and Company) was usually fed, although fresh pig thyroid was used in one series of experiments. The methods employed for feeding and for determining the liver glycogen and the sugar of the blood or urine were the same as described in the first paper,[1] unless otherwise specified.

The Formation of Liver Glycogen in Thyroid-Fed Rats upon a Very High Carbohydrate Diet.

The glycogen content of the liver is rapidly decreased to the minimum by thyroid feeding, and is readily restored by omitting thyroid tissue from the diet. On the other hand, the liver of the thyroid-fed animals apparently does not increase its glycogen content as easily as that of fasted rats after parenteral administration of dextrose.[1] The increased need for energy-producing materials in hyperthyroidism may be the only cause of these phenomena, or it may also be that the glycogenetic or glycogenolytic action of the liver is somewhat affected. Though *in vivo* the

[1] Kuriyama, S., *Am. J. Physiol.*, 1917, xliii, 481.

Digitized by Google

thyroid hormone is thought to accelerate the oxidation of carbohydrates,[2] King[3] reported that *in vitro* the dextrose-destroying power of a mixture of muscle and pancreatic juice (reported as polymerization by others) is markedly retarded by the addition of thyroid juice. Mackenzie,[4] however, observed that thyroid juice has no influence upon blood glycolysis *in vitro*. Opinions as to the glycogenetic function of the liver in some pathological conditions, where the liver glycogen promptly disappears, are still controversial. Grube[5] claims that perfusion of livers with phlorhizin not only prevents formation of glycogen from dextrose, but also breaks down part of the glycogen previously existing. Schöndorff and Suckrow,[6] repeating these experiments, failed to confirm them. According to Neubauer,[7] in phosphorus poisoning as well as in pancreatic diabetes, the liver is affected in its function of forming glycogen from glucose, while this does not hold for levulose. Nishi's experiments,[8] however, show that the glycogenetic function of the liver of depancreatized animals remains normal.

If an increased oxidation is the only or at least the chief cause of the decrease of liver glycogen in hyperthyroidism, a large amount of a very high carbohydrate diet might cause the storage of glycogen in the liver of thyroid-fed animals. Some experiments were made to test this.

Methods.—As the usual food, a paste, made of 70 parts of dog biscuit (rat biscuit which is specially prepared for feeding experiments by Drs. Osborne and Mendel was not used this time) and 30 parts of lard, was given, and as extra carbohydrate, sucrose was added. Giving the dog biscuit-lard diet freely to normal rats, Osborne and Mendel[9] reported that about 4.5 to 9 gm. per day of the food administered to a 150 gm. albino rat or 9 to 13.5 gm. to a 300 gm. rat, are enough to maintain or even to increase the body weight. In my experiments, owing to the frequent decrease of the appetite of the thyroid-fed animals, not much more food than this average could be given. 1 gm. of

[2] Cramer, W., and McCall, R., *Quart. J. Exp. Physiol.*, 1917, xi, 59.

[3] King, J. H., *J. Exp. Med.*, 1909, xi, 665.

[4] Mackenzie, G. M., *J. Exp. Med.*, 1915, xxii, 757.

[5] Grube, K., *Arch. ges. Physiol.*, 1909, cxxviii, 118.

[6] Schöndorff, B., and Suckrow, F., *Arch. ges. Physiol.*, 1911, cxxxviii, 538.

[7] Neubauer, E., *Arch. exp. Path. u. Pharm.*, 1909, lxi, 174.

[8] Nishi, M., *Arch. exp. Path. u. Pharm.*, 1910, lxii, 170.

[9] Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington. Publication No. 156*, pt. i, 1911, 16.

Digitized by Google

desiccated thyroid, mixed with 2 gm. of paste, was given in the afternoon, and after this food was eaten, 10 gm. of paste were put in the cage. On the last day of the experiment, 1 gm. of paste with desiccated thyroid was given at 9 p.m. After this was eaten, 1 gm. of paste and a certain amount of sucrose, mixed with a small amount of water, were given. At 9 the next morning the animal was killed and the liver glycogen determined. As control experiments, a few rats were fasted for 72 hours. At the end of this period, a certain amount of paste or sucrose was given to some of them, the rest still kept fasting. 12 hours later all the animals were killed and the liver glycogen was determined. Neither thyroid-fed, nor control animals showed any diarrhea. The results are detailed in Tables I and II. Rats III, IV, and V in Table II are the same animals which were shown in this *Journal*.[10] The body weight of these three was taken at the end of the 72 hour fasting. In calculating the calories of the food of the last experimental day, the dog biscuit-lard mixture, designated as "paste," was considered to have 5 calories per gm., desiccated thyroid 4.1 calories per gm.

As will be seen in these tables, the storage of glycogen in the liver of thyroid-fed rats is much less than in fasted rats. In some thyroid-fed rats, however, the liver was able to store a noteworthy amount of glycogen. A 72 hour fast decreases the liver glycogen to the minimum; and a small amount of paste or sucrose was enough to make it reappear abundantly within 12 hours. During the same length of time, the thyroid-fed rats ingested several times as many calories as the fasted animals.

Rudinger[11] reported that in Graves' disease the oxidation of protein is abnormally increased and the administration of too much protein accelerates the activity of the thyroid, while the diet with a large amount of carbohydrate has a counter effect, the hyperfunction of the thyroid being retarded by an antagonistic action of the accelerated pancreatic function. According to Cramer and McCall,[2] the oxidation of carbohydrate is also increased in hyperthyroidism. Investigating the respiratory gas

[10] Kuriyama, S., and Mendel, L. B., *J. Biol. Chem.*, 1917, xxxi, 136.
[11] Rudinger, K., *Wien. klin. Woch.*, 1908, xxi, 1581.

Digitized by Google

TABLE I.

The Formation of Liver Glycogen in Thyroid-Fed Rats upon a Very High Carbohydrate Diet.

Rat			I	II	III	IV	V	VI	VII	VIII
Body weight, *gm*			226.5	306.1	215.7	298.9	285.9	273.1	288.5	210.9
Food: Desiccated thyroid, 1 gm., +	1st day.	Paste, *gm*	8.0	6.9	8.0	6.5	8.5	8.9	6.4	8.9
	2nd day.	Paste, *gm*	8.5	6.1	6.5	8.0	6.0	8.6	8.7	6.9
	3rd day.	Paste, *gm*	5.3	7.3	3.9	6.9	7.6	6.7	5.6	8.0
	4th day.	Paste, *gm*	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
		Sucrose, *gm*	3.0	3.0	3.0	8.0	7.0	5.0	6.0	5.0
		Calories	26.4	26.4	26.4	46.9	42.8	34.6	38.7	34.6
Loss of body weight, *gm*			−18.8	−13.2	−12.5	−22.5	−31.7	−29.1	−20.7	−21.0
Liver.	Weight, *gm*		6.8	9.1	5.8	11.0	7.3	6.7	11.1	7.4
	Glycogen content.	*mg*	0	50.0	Trace.	281.1	11.8	36.4	242.8	7.5
		per cent		0.6		2.6	0.2	0.5	2.2	0.1

Digitized by Google

interchange, Magnus-Levy,[12] Thiele and Nehring,[13] and Salomon[14] reported that both healthy men and myxedema patients treated with thyroid substance and also patients with Graves' disease show an increase of 20 to 80 per cent in the amount of oxygen consumed. In experimental hyperthyroidism under the present conditions this increase may be much higher. Although no diarrhea was evident in my experiments, digestibility of a very high carbohydrate diet must also be taken into consideration. The oft reported decrease of the assimilation limit of carbohydrate

TABLE II.

The Formation of Liver Glycogen by Administration of a Certain Amount of Food in Fasted Rats.

Rat			I	II	III	IV	V	VI	VII
Body weight, *gm*			204.0	224.0	150.0	255.0	250.0	207.0	219.5
Food after 72 hour fasting period.	Paste, *gm*.		2.0	0.5	0	0	0	0	0
	Sucrose, *gm*.		0	1.5	2.0	5.0			
	Calories		10.0	8.7	8.2	20.5	0	0	0
Loss of body weight, *gm*			−19.8	−25.7	−30.0	−38.0	−48.0	−24.5	−22.0
Liver.	Weight, *gm*		6.0	6.2	4.2	6.9	4.5	5.3	4.9
	Glycogen content.	*mg*	189.0	322.4	216.1	399.6	Trace.	0	Trace.
		per cent	3.2	5.2	5.2	5.8			

in Graves' disease does not seem to be so great and is not always an accompanying symptom. From my experiments, it will be seen that under suitable conditions, the liver of thyroid-fed rats can store a certain amount of glycogen. Whether the remarkable difference between the thyroid-fed and fasted animals in forming the liver glycogen is due only to an increase of general metabolism cannot yet be clearly answered.

[12] Magnus-Levy, A., *Berl. klin. Woch.*, 1895, xxii, 650; *Deutsch. med. Woch.*, 1896, xxii, 491.

[13] Thiele, O., and Nehring, O., *Z. klin. Med.*, 1896, xxx, 41.

[14] Salomon, H., *Berl. klin. Woch.*, 1904, xli, 635.

Digitized by Google

The Diastase Content of the Liver and Blood Serum of Thyroid-Fed Animals.

Opinions concerning the function of diastase in various animal fluids or tissues and alterations of its concentration in certain pathological conditions are quite controversial. Studying the behavior of the liver diastase in diabetes, Bang[15] reported that the glycogen-free liver of depancreatized dogs showed no excessive diastatic action. He considers this fact as an indication that diabetes depends not on excessive destruction but on deficient formation of glycogen. In the same type of experiments, Zegla[16] found a slight decrease of the liver diastase; but Hinselmann,[17] on the contrary, found an increase. Though Zegla[16] reported that the diastase content of the liver increases in phlorhizin or phloretin glycosuria, the slight difference in the results of his experiments seems still to be within the technical error or physiological variation. Wohlgemuth and Benzur[18] reported that phlorhizin, phloretin, or epinephrine injection causes an increase of the diastase content of the kidneys, while the diastase content of the liver and blood serum shows no decided change. Wohlgemuth[19] demonstrated also that fasting, change of diet, increase of pancreatic activity by hydrochloric acid or secretin have no effect upon the diastase content of the blood. Knowing that epinephrine, *piqûre*, and other nervous influences are without action upon the blood diastase, Moeckel and Rost[20] are of the opinion that the blood diastase is a product of the metabolism of the cells, which has no special significance and serves no function in the organism. Macleod and Pearce[21] reported that in an etherized animal after death there is usually, though not always, an acceleration in the rate of glycogenolysis of the liver. Studying the glycogenolytic power of the liver after splanchnic stimulation, the same authors[22] concluded that modifications in the glycogenolytic activity of the liver do not depend on changes in the amount of diastase, but on changes in the conditions under which a constant amount of this ferment is acting. Scaffidi[23] demonstrated that the liver of frogs poisoned with phosphorus has a glycogenolytic power as high as the normal liver. Bang[24] asserts that the liver of *Rana esculenta* contains a large amount of

---

[15] Bang, I., *Beitr. chem. Physiol. u. Path.*, 1907, x, 320.

[16] Zegla, P., *Biochem. Z.*, 1909, xvi, 111.

[17] Hinselmann, H., *Z. physiol. Chem.*, 1909, lxi, 265.

[18] Wohlgemuth, J., and Benzur, J., *Biochem. Z.*, 1909, xxi, 460.

[19] Wohlgemuth, J., *Biochem. Z.*, 1909, xxi, 381.

[20] Moeckel, K., and Rost, F., *Z. physiol. Chem.*, 1910, lxvii, 433.

[21] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1910–11, xxvii, 341.

[22] MacLeod and Pearce, *Am. J. Physiol.*, 1911, xxviii, 403.

[23] Scaffidi, V., *Biochem. Z.*, 1915, lxviii, 320.

[24] Bang, *Biochem. Z.*, 1913, xlix, 40.

Digitized by Google

latent diastase, which does not act in the physiological formation of sugar in the liver, while the liver of *Rana fusca* contains only a small amount of such latent diastase. In human diabetes, Wynhausen[25] failed to demonstrate any decided change of the blood diastase, while Myers and Killian's recent studies[26] showed a noteworthy increase.

Feeding thyroid to cats, Burge, Kennedy, and Neill[27] found that the catalase content of the blood increases markedly. On the other hand, Juschtschenko[28] reported that thyroidectomy causes a decrease of the catalase content of the blood. Wells[29] sought for a possible influence on autolysis of the liver by thyroid extract, but could demonstrate none *in vitro*. Schryver,[30] however, found that autolysis was more rapid in the liver of dogs fed on thyroid extract for some days before death than it was in control animals.

Concerning the influence of the thyroid upon the blood diastase, Wohlgemuth[19] found a noteworthy decrease of the blood diastase in thyroidectomized goats. The diastase content of the blood serum of these animals was $D_{\frac{38°}{2h}} = 3.3 - 5$, while the normal goat serum was valued at $D_{\frac{38°}{2h}} = 20 - 40$.

I have made some experiments in order to ascertain whether the decrease of the glycogen content of the liver in hyperthyroidism has any relation to the glycogenolytic action of the liver and blood serum.

Records of these follow.

Methods.—Rabbits were used for blood diastase determination; rats for liver diastase. To the former, fresh pig thyroid was given; to the latter, desiccated thyroid. In rabbits, the blood was taken from an ear vein by puncture, and the serum separated. For determining the liver diastase, the organ was extirpated promptly after bleeding the animals and immersed in warmed physiological saline solution. After weighing, the liver was freed from blood by perfusing with the same saline solution. The liver was then thoroughly ground in a mortar with four times its weight of physiological saline solution and a small amount of sand, placed in an ice box for 1 hour, and shaken from time to time. After filtration through a cloth, the homogeneous organ

[25] Wynhausen, O. J., *Berl. klin. Woch.*, 1910, xlvii, 1281.

[26] Myers, V. C., and Killian, J. A., *J. Biol. Chem.*, 1917, xxix, 179.

[27] Burge, W. E., Kennedy, J., and Neill, A. J., *Am. J. Physiol.*, 1917, xliii, 433.

[28] Juschtschenko, A., *Biochem. Z.*, 1910, xxv, 49.

[29] Wells, H. G., *Am. J. Physiol.*, 1904, xi, 351.

[30] Schryver, S. B., *J. Physiol.*, 1904–05, xxxii, 159.

Digitized by Google

emulsion was used for diastase determination by Wohlgemuth's method.[18, 31] The results are shown in Tables III and IV.

TABLE III.

The Diastase Content of the Blood Serum of Thyroid-Fed Rabbits.

Rabbit.....	I	II	III*	IV†
Body weight, *kg*.....	3.30	2.64	2.86	2.58
Food.....	Oats.	Oats.	Oats + greens.	Oats + greens.
Fresh pig thyroid, 15 gm. per day.....	June 8–11	June 13–16	June 8–11	June 8–11
Diastase content of serum. $D_{24h}^{37°}$ =	62.5 May 25	62.5 June 12	62.5 May 24	62.5 May 24
	62.5 June 10	62.5 " 15	62.5 " 25	62.5 June 10
	62.5 " 11	62.5 " 15	62.5 June 11	100.0 " 11
	62.5 " 12	62.5 " 16	100.0 " 12	62.5 " 12

* Died on June 14. † Died on June 12.

TABLE IV.

The Diastase Content of the Liver of Thyroid-Fed Rats.

Thyroid-Fed Rats. Food, Desiccated Thyroid, 1 Gm. per Day; Paste, 4 Gm. per Day; Dog Biscuit, Freely; for 4 Days.

Rat.....		I	II	III	IV	V
Body weight, *gm*.....		128.1	162.9	127.2	109.0	133.0
Liver.	Weight, *gm*.....	7.4	9.3	6.4	4.6	5.0
	Diastase content, $D_{24h}^{37°}$ = ...	156	250	250	156	250

Control Rats. Food, Paste, 5 Gm. per Day; Dog Biscuit, Freely; for 4 Days.

Rat.....		I	II	III	IV
Body weight, *gm*.....		117.3	148.4	219.0	301.4
Liver.	Weight, *gm*.....	4.4	6.1	9.4	10.3
	Diastase content, $D_{24h}^{37°}$ = ...	156	156	250	250

[31] Wohlgemuth, *Biochem. Z.*, 1908, ix, 1.

Digitized by Google

As the diastase content of the blood serum of normal rabbits, Wohlgemuth and Benzur[18] obtained the value of $D_{24}^{35°} = 62.5$, which sometimes increased to 100 or 150. In my normal rabbits, the blood serum always showed the value of $D_{24}^{35°} = 62.5$. After thyroid feeding, the diastase content of the blood serum remained about the same. As to the diastase content of the liver, the animal species seem to show some noteworthy difference. In rabbits it is said to vary between $D_{24}^{35°} = 5$ and 12.5 (Wohlgemuth and Benzur[18]), while in rats $D_{24}^{35°}$ is as high as 80 (meat feeding) or 160 to 320 (bread or lard feeding) (Hirata[32]). In my own experiments, the diastase content of the normal rat liver was $D_{24}^{35°} = 156$ to 250. This accords well with Hirata's results. In thyroid-fed rats, the value was about the same. As far as the diastase content of the blood serum and the liver is concerned, the decrease of the glycogen content of the liver in experimental hyperthyroidism does not seem to be due to an increased glycogenolytic power of the liver or blood.

The diastase content, however, is always determined on the dead material. The diastatic power of such substances may not be identical with that in the living condition. Comparing the diastatic efficiency and average glycogen content in the different tissues and organs, MacLean[33] reported that the diastatic power and the glycogen content do not necessarily run parallel and sometimes an organ containing merely a trace of glycogen, *e.g.*, the lung, shows a more marked amylolytic power than a glycogen-rich organ, such as the liver. Moreover, a long list of substances, which accelerate or inhibit the activity of diastase, has been reported. A small amount of such substances within the living cells may markedly affect the activity of this enzyme. It is a well known fact that a marked increase of protein and fat metabolism exists in hyperthyroidism. The nitrogenous constituents of the urine are usually increased. Bock[34] reported an increase of amino-acid nitrogen of the blood in a case of hyperthyroidism (9.58 mg. amino-acid N in 100 cc. of blood; the nor-

[32] Hirata, G., *Biochem. Z.*, 1910, xxvii, 385.
[33] MacLean, H., *Biochem, J.*, 1909, iv, 467.
[34] Bock, J. C., *J. Biol. Chem.*, 1917, xxix, 191.

Digitized by Google

mal blood containing only 7.13 mg.). Many cleavage products of protein, such as amino-acids, creatine, creatinine, and others, have been reported to accelerate the activity of the diastase.[35] Though the liver and blood of thyroid-fed animals do not show any increased diastase content, it may be possible that some substances, which are produced to an abnormally large degree, on account of an increase of metabolism, accelerate the activity of diastase within the living cells.

The Influence of Thyroid Feeding upon the Glycogen Content of the Liver of Full Grown Frogs.

Determining the glycogen content of the frog (*Rana fusca*) at different seasons, Athansiu[36] reported that the glycogen content of the frog body is lowest in the summer, at which time they take the most nourishment, and the highest in the fall, gradually decreasing in the winter. At the end of hibernation the body still contains a large amount of glycogen. This statement was confirmed in general by Pflüger.[37] In frogs, glycosuria can be induced by subcutaneous application of phlorhizin, but not by oral administration.[38] Scaffidi[23] reported that phosphorus poisoning induces a marked loss of the liver glycogen of frogs (*Rana esculenta*) in a short time. Mangold,[39] however, observed that after the long continued convulsions caused by strychnine, which promptly reduce the liver glycogen of warm-blooded animals, the liver of a frog still contained a large amount of glycogen. As shown by Gudernatsch,[40] Lenhart,[41] and Graham,[42] thyroid feeding exerts a remarkable influence upon the growth of tadpoles. Emaciation, retardation in gain of body weight, and early differentiation of body parts can be noticed in a few days. Lenhart considers that the effect of thyroid feeding is closely associated with both the iodine content of thyroid and the amount fed.

In full grown frogs, the metabolism is probably most active in the summer time. It may be interesting to know whether in this season thyroid feeding of full grown frogs can increase the metabolism or at least cause any decrease of the glycogen con-

[35] Effront, J., *Compt. rend. Soc. biol.*, 1904, lvii, 234. Terroine, E. F., and Weill, J., *J. physiol. et path. gen.*, 1912, xiv, 437.
[36] Athansiu, J., *Arch. ges. Physiol.*, 1899, lxxiv, 561.
[37] Pflüger, E., *Arch. ges. Physiol.*, 1907, cxx, 253.
[38] Lusk, G., *Ergebn. Physiol.*, 1912, xii, 315.
[39] Mangold, E., *Arch. ges. Physiol.*, 1907–08, cxxi, 309.
[40] Gudernatsch, J. F., *Am. J. Physiol.*, 1914–15, xxxvi, 370.
[41] Lenhart, C. H., *J. Exp. Med.*, 1915, xxii, 739.
[42] Graham, A., *J. Exp. Med.*, 1916, xxiv, 345.

Digitized by Google

TABLE V.

The Glycogen Content of the Liver of Thyroid-Fed Frogs. Desiccated Thyroid, 1 Gm. per Day.

Frog		I	II	III	IV	V
Body weight, *gm.*		214.7	183.3	234.3	144.3	133.8
Duration of thyroid feeding, *days*		3	6	8	6	6
Change of body weight, *gm.*		−3.6	+2.2	−9.0	+7.1	−3.2
Liver.	Weight, *gm.*	4.5	6.1	5.3	4.1	3.6
	Glycogen content. *mg.*	156.3	372.8	229.0	215.5	175.5
	Glycogen content. *per cent.*	3.5	6.1	4.3	5.3	4.9

Control Frogs.

Frog	I	II	III	IV
Body weight, *gm.*	195.9	133.3	195.6	136.9
Food	No food for 3 days.	No food for 6 days.	Powdered egg, 1 gm. per day, for 8 days.	Powdered egg, 1 gm. per day, for 6 days.
Change of body weight, *gm.*	−1.8	0	+2.6	+7.8
Liver. Weight, *gm.*	4.5	3.2	3.9	3.9
Glycogen content. *mg.*	274.0	117.2	104.0	151.2
Glycogen content. *per cent.*	6.1	3.7	2.7	3.9

Digitized by Google

tent of the liver. Some experiments bearing upon this point are recorded below.

Method.—The experiments were carried on with *Rana pipiens* in June. Desiccated thyroid, made into a paste by mixing with a small amount of water, was given orally. The dose of desiccated thyroid was 0.5 gm., twice daily. The animals were kept in running tap water and no food was given to them. The control frogs were divided into two groups. One group was kept in running water only. To the other group, boiled, desiccated, and powdered egg was fed, in quantities comparable to those of the thyroid given. The results are detailed in Table V.

As shown in this table, there is no decided difference in the glycogen content of the livers of thyroid-fed frogs and those of control animals. The glycogen content of the liver of thyroid-fed frogs was 4.8 per cent (the average of 5 cases), while in the control frogs it was 4.1 per cent (the average of 4 cases). In none of the animals was any noteworthy change of the general conditions observed. Under the experimental conditions employed, thyroid feeding does not decrease the glycogen content of the liver. This is in harmony with the result obtained in a frog poisoned with strychnine and seems to indicate a great difference in the intensity of metabolism in the different stages of the life of the frog.

SUMMARY.

The storage of glycogen in the liver of thyroid-fed rats does not occur readily, even with the administration of a large amount of a very high carbohydrate diet. In fasted rats the liver glycogen reappears abundantly after ingestion of a comparatively small amount of food. If a sufficiently large calorific food intake is administered to thyroid-fed rats, liver glycogen may sometimes reappear to a limited extent. The quantity of glycogen so stored is, however, much smaller than that in fasted rats, subsequently given food with a fuel value several times less than that of the thyroid-fed rats.

The diastase content ($D_{M}^{37.5}$) of the normal rabbit serum and the normal rat liver is about 62.5 and 156 to 250 respectively. Thyroid feeding does not change these values markedly.

Digitized by Google

The glycogen of the liver of thyroid-fed frogs (*Rana pipiens*) is practically the same as that of control animals. This accords with the results obtained with frogs poisoned with strychnine or in hibernation and perhaps indicates a notable difference in the intensity of metabolism of warm- and cold-blooded animals and of the frog at different life stages.

Digitized by Google

Digitized by Google

THE INFLUENCE OF THYROID FEEDING UPON CARBOHYDRATE METABOLISM. II.

THE EPINEPHRINE CONTENT OF THE ADRENALS OF THYROID-FED RATS.

By SHIGENOBU KURIYAMA.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, November 26, 1917.)

In my previous investigations,[1] the epinephrine content of the adrenals of white rats, fed on thyroid for a few days, was little different from that of control animals. A colorimetric method of estimation with mercuric acetate was used. Folin, Cannon, and Denis[2] reported that phosphotungstic acid (uric acid reagent) is a very sensitive reagent, solutions containing one part of epinephrine in 3,000,000 parts of water producing an unmistakable reaction with this reagent, and therefore being approximately ten times as sensitive as other chemical tests. Using Folin's method, Herring[3] reported that the administration of raw thyroid to cats and rats can increase the epinephrine content of the adrenals. The adrenals were also heavier than those of control animals. Hoskins[4] studied the influence of thyroid feeding upon the adrenal glands. In new-born guinea pigs, the adrenal glands were 25 per cent heavier than those of control animals. In young white rats this phenomenon was also confirmed. The heart, spleen, liver, and kidneys were also heavier than those of control animals.

The present experiments extend previous observations with the white rats.

Method.

Albino rats were divided into three series, each having nearly the same number of each sex. The body weight of each series averaged about the same. All of them were fed on dog biscuit-

[1] Kuriyama, S., *Am. J. Physiol.*, 1917, xliii, 481.

[2] Folin, O., Cannon, W. B., and Denis W., *J. Biol. Chem.*, 1912-13, xiii, 477.

[3] Herring, P. T., *Quart. J. Exp. Physiol.*, 1915–16, ix, 391; 1917, xi, 47.

[4] Hoskins, R. G., *J. Am. Med. Assn.*, 1910, lv, 1724. Hoskins, E. R., *J. Exp. Zool.*, 1916, xxi, 295.

Digitized by Google

lard diet. Two series were used for thyroid feeding, the third one serving as control. Each rat was kept in a separate cage. As thyroid administration 0.1 gm. of desiccated thyroid per day was prescribed to each rat of the one series, and this was continued for 23 days. Every rat of the other series received 1 gm. of desiccated thyroid per day for 4 days. Thyroid powder, mixed with two or three times the weight of the dog biscuit-lard paste, was readily eaten. After this had been consumed, the paste was given freely. At the end of the period, the animal was bled and both adrenals were carefully freed from the surrounding tissues and weighed together. The epinephrine content was determined by Folin's method, modified for smaller quantities of material. The protein of the ground adrenals was precipitated by acid and salt in a 10 cc. volumetric flask. The mixture was diluted up to the mark and filtered. 8 cc. of the filtrate were taken into a 25 cc. volumetric flask and the color, produced in this flask, was compared with the standard color in another 25 cc. flask. For the standard color, two 25 cc. volumetric flasks containing 0.2 and 0.4 mg. of uric acid, respectively, were provided and the flask which was the nearer in color to the epinephrine flask was used. The results are shown in Tables I and II.

TABLE I.

The Epinephrine Content of the Adrenals of Normal Rats.

Rat.	Sex.	Body weight.	Weight of two adrenals.	Weight of two adrenals per 100 gm. of body weight.	Epinephrine in two adrenals.	Epinephrine per gm. of adrenal.	Epinephrine per 100 gm. of body weight.
		gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	M.	304.8	36.9	12.1	0.102	2.77	0.033
II	"	269.7	35.3	13.1	0.094	2.66	0.035
III	"	254.3	63.1	24.8	0.162	2.57	0.064
IV	"	218.1	57.5	26.4	0.099	1.72	0.045
V	"	180.0	33.8	18.8	0.091	2.69	0.051
VI	F.	243.8	50.6	20.8	0.134	2.65	0.055
VII	"	240.2	50.6	21.1	0.174	3.44	0.072
VIII	"	199.4	39.5	19.8	0.098	2.48	0.049
IX	"	196.2	39.5	20.1	0.104	2.63	0.053
X	"	194.6	39.1	20.1	0.100	2.56	0.051
XI	"	69.2	25.4	36.7	0.056	2.20	0.081
Average.....		**215.5**	**42.8**	**21.3**	**0.110**	**2.58**	**0.064**

Digitized by Google

TABLE II.

The Epinephrine Content of the Adrenals of Thyroid-Fed Rats.

Rat.	Sex.	Change of body weight during experiment.	Body weight at end of experiment.	Weight of two adrenals.	Weight of two adrenals per 100 gm. of body weight.	Epinephrine in two adrenals.	Epinephrine per gm. of adrenal.	Epinephrine per 100 gm. of body weight.
Series A. Desiccated thyroid; 1 gm. per day for 4 days.								
			gm.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
I	M.	−17.2	276.4	42.0	15.2	0.095	2.26	0.034
II	"	−18.5	254.2	34.1	13.4	0.092	2.70	0.036
III	"	−23.0	244.0	34.8	14.3	0.093	2.67	0.038
IV	"	−14.9	126.9	28.1	22.1	0.059	2.10	0.047
V	"	−22.9	125.4	30.5	24.3	0.057	1.87	0.045
VI	F.	−17.0	267.8	44.9	16.8	0.092	2.05	0.034
VII	"	−18.8	189.9	48.3	25.4	0.106	2.20	0.056
VIII	"	−17.3	182.8	43.4	23.7	0.111	2.56	0.061
IX	"	−19.1	178.6	64.5	36.1	0.119	1.85	0.067
X	"	−18.8	178.1	43.4	24.4	0.084	1.94	0.047
XI	"	−17.8	173.4	44.3	25.5	0.101	2.28	0.058
Average.....			**199.8**	**41.7**	**21.9**	**0.092**	**2.23**	**0.048**
Series B. Desiccated thyroid; 0.1 gm. per day for 23 days.								
I	M.	−2.8	267.3	50.0	18.7	0.101	2.02	0.038
II	"	−5.4	231.6	41.5	17.9	0.098	2.36	0.042
III	"	−10.0	221.0	46.6	21.1	0.126	2.70	0.057
IV	"	−2.9	210.7	36.2	17.2	0.071	1.96	0.034
V	F.	−4.8	198.4	50.6	25.5	0.142	2.81	0.072
VI	"	+0.9	176.4	38.4	21.8	0.082	2.14	0.047
VII	"	−2.6	149.4	37.8	25.3	0.083	2.20	0.056
VIII	"	−14.1	144.6	42.1	29.1	0.081	1.92	0.056
Average.....			**199.9**	**42.9**	**22.1**	**0.098**	**2.26**	**0.050**

As the epinephrine content of the adrenals of normal animals (mg. of epinephrine per gm. of the gland), Folin and his coworkers[2] found 2.41 to 3.08 mg. in the sheep, 2.04 to 2.33 mg. in the dog, 3.33 to 3.52 mg. in the calf, 1.22 to 1.52 mg. in the young cat, and Herring found 1.25 to 2.22 mg. in the cat, and 1.47 to 2.97 mg. in the albino rat. In my experiments this value in albino rats averaged 2.20 mg. The maximum was 3.44

Digitized by Google

mg., the minimum 1.72 mg. It will be seen that the epinephrine content of the adrenal shows some variation according to both the animal species and the individual.

As the thyroid hormone is considered to stimulate the sympathetic nervous system, an excess of it in the body may stimulate the activity of the adrenal. But this may not necessarily be accompanied by a higher content of the epinephrine in the gland. The excess of epinephrine may immediately be transported into the circulation, its concentration in the gland being kept as usual. It is also possible that the epinephrine produced in the gland, no matter what the amount may be, is quickly expelled by the abnormal nerve stimulation and consequently the lower concentration soon manifests itself. Quinquaud[5] reported that the epinephrine content of the adrenal was decreased after *piqûre*. Confirming the results of Elliott,[6] Stewart and Rogoff[7] reported that diminution of the stock of epinephrine in the adrenal, through electrical stimulation of the splanchnics, was not easy to demonstrate, despite the fact that the liberation of epinephrine into the blood was notably increased by the stimulation. Only after a stimulation, repeated at intervals for a long time, could a distinct depletion of the epinephrine content be observed. The results in Table II show that the epinephrine content of the adrenal was practically not changed by thyroid feeding (a small dose per day for a long time, and a large dose per day for a short time), neither absolutely nor relatively, the individual or physiological variations being kept in mind. Neither do the absolute and relative weights of the adrenals show any noteworthy difference from those of the control animals. According to Donaldson's tables,[8] the weight of the adrenals of albino rats is 45.6 mg. (average of both sexes) for 200 gm. of body weight, and 48.2 mg. (average of both sexes) for 215 gm. of body weight. In his chart it may be seen that there exists a widely ranging variation. In Herring's report, the epinephrine content of the adrenals of the thyroid-fed animals was increased absolutely but decreased when it was calculated per gm. of the adrenal. The weight of the fresh adrenals

[5] Quinquaud, A., Relations entre la piqûre diabétique et la sécrétion d'adrénaline, Paris, 1915.

[6] Elliott, T. R., *J. Physiol.*, 1912, xliv, 374.

[7] Stewart, G. N., and Rogoff, J. M., *J. Exp. Med.*, 1916, xxiv, 709.

[8] Donaldson, H. H., The Rat, Philadelphia, 1915, 100, 137.

Digitized by Google

was increased. On the other hand, the desiccated adrenal of the thyroid-fed animals had a higher adrenal content than the same amount of desiccated normal adrenal. This seems to show that the increase of the adrenal weight was mostly due to the water content and not to the solid substance. Obtaining the blood of the adrenal vein of dogs, into which extracts of various organs had been previously injected, and testing the vasoconstrictor action of this blood sample with another dog, Gley and Quinquaud[9] concluded that though the extracts of various organs can augment the secretion of epinephrine, the extract of thyroid gland is not specific. Gley's other experiments[10] demonstrate that the epinephrine content of the adrenal of thyroidectomized dogs and rabbits is not lower than that of normal animals. This is not in harmony with the results of Herring with thyroidectomized cats.[3]

Though an augmented secretion of epinephrine in hyperthyroidism may be probable, the frequently reported intimate relation between the hormones of the thyroid and adrenal has also been studied from different standpoints. Macleod and Pearce[11] observed that after complete dissection of the hepatic plexus, stimulation of the splanchnic nerve is only occasionally followed by an increase in the sugar content of the vena cava blood, although a marked hyperglycemia is found in cases of the intact hepatic plexus. They therefore concluded that there was no evidence that hyperglycemia, caused by stimulation of splanchnic nerve, is due to a hypersecretion of epinephrine. Oswald[12] demonstrated that though intravenous injection of iodothyreoglobulin has no effect on the circulation, this treatment increases the effectiveness of epinephrine in raising and maintaining blood pressure. Asher,[13] Richardson,[14] Kakehi,[14] and Eiger[15] reported that

[9] Gley, E., and Quinquaud, A., *Arch. Internat. Physiol.*, 1914, xiv, 152.

[10] Gley, E., *Arch. Internat. Physiol.*, 1914, xiv, 175.

[11] Macleod, J. J. R., and Pearce, R. G., *Am. J. Physiol.*, 1911–12, xxix, 419.

[12] Oswald, A., *Z. Physiol.*, 1915, xxx, 509; cited by Levy.[16]

[13] Asher, L., *Deutsch. Med. Woch.*, 1916, xxxiv, 1; abstr. in *Physiol. Abst.*, 1916–17, i, 426.

[14] Richardson, H. B., *Z. Biol.*, 1916, lxvii, 57. Kakehi, S., *ibid.*, 1916, lxvii, 104; both papers abstr. in *Physiol. Abst.*, 1916–17, i, 427.

[15] Eiger, M., *Z. Biol.*, 1917, lxvii, 253, 265; abstr. in *Physiol. Abst.*, 1917, ii, 269.

Digitized by Google

in the experiments with Laewen-Trendelenburg frog or rabbit heart, the action of epinephrine was augmented by the addition of thyroid extract. In the same manner, the plasma of rats fed on thyroid and plasma obtained from patients with Graves' disease, had a well marked accelerating action on vasoconstrictor influence of epinephrine. Asher considers that thyreoglandol, which is protein-free and practically iodine-free, exerts the same biological and metabolic action as the whole gland. Levy[16] reported that in ordinary and adrenalectomized cats stimulation of the cervical sympathetic nerve can increase the effectiveness of epinephrine in raising arterial pressure, although this is not the case in thyroidectomized cats. He concluded that thyroid secretion renders more excitable the sympathetic structures acted on by epinephrine in raising arterial pressure, and the increased effectiveness of epinephrine as a pressor agent after thyroid stimulation is not dependent on a greater amount of circulating epinephrine. On the other hand, however, he and Cannon and Cattell[17] obtained some evidence indicating that stimulation of the cervical sympathetic nerve or epinephrine injection induces secretory activity in the thyroid gland. In my previous experiments,[1] when epinephrine was injected into thyroid-fed rabbits, epinephrine glycosuria was induced to the same extent as in control animals; the hyperglycemia being practically the same or somewhat augmented.

SUMMARY.

The epinephrine content of the adrenals of normal medium sized albino rats is 2.2 mg. per gm. of the gland. Comparing this figure with those reported by other investigators with larger animals, the epinephrine content of rat adrenal is the nearest to that of the dog, larger than that of the cat, and smaller than that of the sheep and calf. Thyroid feeding of either short duration with large doses or long duration with small doses does not materially change the epinephrine content, nor the weight of the adrenals of medium sized albino rats. If hypersecretion of the adrenals really exists in experimental hyperthyroidism, these

[16] Levy, R. L., *Am. J. Physiol.*, 1916, xli, 492.
[17] Cannon, W. B., and Cattell, McK., *Am. J. Physiol.*, 1916, xli, 74.

Digitized by Google

results indicate that the excess of epinephrine is promptly transported into the circulation, the epinephrine content of the adrenals being kept fairly constant. The presence of such a regulatory mechanism might well be expected from the experiments of Elliott and Stewart and Rogoff.

Digitized by Google

Digitized by Google

THE INFLUENCE OF THYROID FEEDING UPON CARBOHYDRATE METABOLISM. III.

THE ACIDOSIS IN EXPERIMENTAL HYPERTHYROIDISM AND ITS RELATION TO EPINEPHRINE IN THE BLOOD AND THE DECREASE OF LIVER GLYCOGEN.

By SHIGENOBU KURIYAMA.

(*From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.*)

(Received for publication, November 26, 1917.)

The Influence of Thyroid Feeding upon the Hydrogen Ion Concentration and the Alkali Reserve of the Blood Plasma.

The influence of acid and alkali upon carbohydrate metabolism, with respect to glycosuria, blood sugar content, and glycogen content of the liver, has been discussed by many investigators. Edie and his coworkers[1] reported that the glycosuria found after partial asphyxiation is due to the high percentage of carbon dioxide in the respired air. MacLeod[2] demonstrated that hyperglycemia and glycosuria in asphyxia and curare poisoning are due to the mobilization of liver glycogen and that here the blood acts directly upon the liver cells without any intermediation of the nervous system. Estimating the rate of disappearance of glycogen from pieces of liver, both in an atmosphere of carbon dioxide and in air or oxygen, he also found that glycogenolysis is more rapid in the former than in the latter. Administering hydrochloric acid by mouth to rabbits and also perfusing turtle livers with a solution acidified with hydrochloric acid, Elias[3] demonstrated that a relatively small amount of acid can mobilize the glycogen of the liver and cause hyperglycemia and glycosuria. Studying the adrenal histologically, he considered that these glands have no direct relations to the glycogen mobilization caused by acid. Elias and Kolb[4] have also concluded that the so called "hunger diabetes" of young dogs, is, in part at least, a condition due to acidosis, as indicated by the lowered carbon dioxide content of the blood and of the alveolar air. The word

[1] Edie, E. S., *Biochem. J.*, 1906, i, 455. Edie, E. S., Moore, B., and Roaf, H. E., *ibid.*, 1911, v, 325.

[2] Macleod, J. J. R., *Am. J. Physiol.*, 1908–09, xxiii, 278.

[3] Elias, H., *Biochem. Z.*, 1913, xlviii, 120.

[4] Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.

Digitized by Google

acidosis is used to express an actual increase in the hydrogen ion concentration of the blood as well as a lowering of the alkali reserve. It has been pointed out that free carbon dioxide is present in the body fluids in such concentration that it automatically converts into bicarbonates all bases not bound by other acids. In acidosis the concentration of bicarbonate in the blood is always reduced below the normal level. As a sensitive indicator of this condition and its severity, the carbon dioxide capacity of the plasma was determined by Van Slyke and Cullen.[5]

Since thyroid feeding can decrease the glycogen content of the liver very easily, possibly acidosis plays some rôle here.

Methods.

Full grown rabbits were used. 3 gm. of desiccated thyroid, suspended in water, were given by a stomach sound every afternoon. Oats or greens (cabbage and carrot tops) were given as food. As shown by Sherman and Gettler,[6] and McDanell and Underhill,[7] the kind of food has a marked influence upon the acid-base equilibrium of the organism. Attention to this was, therefore, paid both in the preparatory and the thyroid period. When greens were given, the thyroid-fed rabbits continued to take them until the later part of the thyroid period in about the same quantities as in the preparatory period. In the case of the oat diet, the appetite of the animal was greatly affected by thyroid feeding. They stopped eating this food in a few days. As control experiments, therefore, rabbits were fasted and others were fed on desiccated animal tissue other than thyroid, no other food being added. As such a tissue powder, a mixture of desiccated spleen, kidney, and parotid gland in equal proportions was used. The amount and the method of administration of this powder were the same as in thyroid feeding. The urine was examined for protein and casts from time to time. The hydrogen ion concentration of the blood was determined by Marriott's method.[8] 1 cc. of oxalated venous blood was used for dialysis. After comparing the color, produced by phenolsulfonephthalein, with the standard colors, the dialysate was

[5] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 289.
[6] Sherman, H. C., and Gettler, A. O., *J. Biol. Chem.*, 1912, xi, 323.
[7] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 233.
[8] Marriott, W. McK., *Arch. Int. Med.*, 1916, xvii, 840.

Digitized by Google

vigorously aerated, and the reaction thus changed, which now served as an index of the alkali reserve of the blood, was again estimated colorimetrically. The CO_2 capacity of the plasma was determined by Van Slyke and Cullen's method,[5] the larger size apparatus[9] being used. The results were calculated so that they showed cc. of CO_2 reduced to 0°C., and 760 mm. Hg, bound as bicarbonate in 100 cc. of the plasma. The blood was collected from the external jugular vein by a needle connecting to a cannula passing to the bottom of a centrifuge tube containing potassium oxalate and a layer of paraffin oil. No anesthetic was used. During the operation the rabbit was kept quiet so as not to disturb the circulation and respiration. 8 or 9 cc. of the venous blood were collected at once. After mixing with the oxalate, part of it was taken out for dialysis and the rest was centrifuged for obtaining the plasma. The same vein could be used for several punctures. But if thrombosis occurred, the vein on the other side was used. The blood was examined usually in the morning. When this was done twice a day, the first examination was made in the morning and the second one in the afternoon after thyroid feeding. On the first experimental day, the blood examination was made before changing foodstuffs or before the first thyroid feeding. The results of this day, therefore, can be considered the normal value. The results are shown in Tables I and II, and Charts I and II.

From these results it will be seen that the hydrogen ion concentration of the normal rabbit blood is pH 7.35 to 7.5. The kind of food caused no noteworthy difference. After aeration of the dialysate the value became pH 8.1 to 8.2. Levy and his coworkers[10] reported that pH of the oxalated blood from normal men varies from 7.4 to 7.6. This is nearly the same as my results with the rabbit blood. When thyroid was given to the oat-fed rabbits, the hydrogen ion concentration of the blood slightly increased (the maximum, pH 7.25 to 7.3). In the blood of acidosis patients, Levy and his coworkers[10] observed a reaction of pH 7.1 to 7.4; therefore nearly the same degree of acidosis as found in my thyroid-fed rabbits. A slight decrease of the

[9] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

[10] Levy, R. L., Rowntree, L. G., and Marriott, W. McK., *Arch. Int. Med.*, 1915, xvi, 389.

Digitized by Google

TABLE 1.

The Influence of Thyroid Feeding upon the Hydrogen Ion Concentration of the Blood and the Alkali Reserve of the Blood Plasma.

Series A. Oats Given Freely.

Experimental day.	Body weight.	Desiccated thyroid.	Oats.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit I. (Before experiment oats only were given freely for 5 days.)								
	kg.	*gm.*	*gm.*				*cc.*	
1	3.06	3	60	7.45	8.1	52.2	116	Urine: protein −, casts −.
2		3	14	7.45	8.1	48.0	130	
3		3	0	7.35	8.0	45.2	100	
4		3	0	7.35	8.0	40.9	106	
5		3	0	7.30 7.25	7.9 7.9	30.5 28.7	52	Urine: protein +, casts +. Died on the 6th day.
Rabbit II. (Before experiment oats only were given freely for 5 days.)								
1	2.48	3	52	7.45	8.1	53.2	93	Urine: protein −, casts −.
2		3	38	7.45	8.1	57.0	80	
3		3	10	7.45	8.1	57.9	65	
4		3	0	7.45	8.1	47.0	69	
5	2.09	0	0	7.25 7.25	8.0 8.0	29.6 29.6	81	Urine: protein ±, casts −.
6		0	Oats and	7.45	8.1	29.6	78	
7		0	greens were	7.45	8.1	43.8	102	
8	2.13	0	given freely.	7.45	8.1	60.7	99	Recovered.
Rabbit III. (Before experiment oats only were given freely for 5 days.)								
1	1.62	3	43	7.35	8.1	51.0	87	Urine: protein −, casts −.
2		3	5	7.35	8.1		151	
3		3	0	7.35	8.1	44.3	131	
4		3	0	7.35	8.1	43.3	126	
5		3	0	7.35	8.1	43.2	92	Urine: protein −, casts −.
6	1.30	3	0	7.35	8.1	37.5	82	
7		0	0	7.3	8.1	29.5	?	Died on 8th day.
Rabbit IV. (Before experiment oats and greens were given freely for 5 days.)								
1	2.10	3	34	7.5	8.2	62.3	154	Urine: protein −, casts −.
2		3	24				140	
3		3	0	7.4	8.2	42.9	?	
4	1.92	3	0				91	Urine: protein ±, casts −.
5		3	0	7.35	8.1	30.3	82	Died on 6th day.

Digitized by Google

TABLE I—*Concluded.*

Series B. Greens Given Freely.

Experimental day.	Body weight.	Desiccated thyroid.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit I. (Before experiment greens only were given freely for 5 days.)							
	kg.	*gm.*				*cc.*	
1	3.02	3	7.45	8.1	63.6	305	Urine: protein −, casts −.
2		3	7.45	8.2	63.6	410	
3		3	7.45	8.1	60.7	350	
4		3	7.45	8.1	55.3	400	
5		3	7.45	8.1	46.6	292	
6		3	7.45	8.1	48.5	306	
7		3	7.45	8.1	52.2	320	Urine: protein −, casts −.
8	2.20	0	7.45	8.1	43.8	380	Recovered by stopping thyroid feeding.
Rabbit II. (Before experiment greens only were given freely for 3 days.)							
1	1.34	3	7.5	8.1	61.4	320	Urine: protein −, casts −.
2		3				329	
3		3				420	
4		3	7.45	8.1	49.0	266	Urine: protein ±, casts −.
5	0.98	3	7.45	8.1	46.2	408	Died on 6th day.

alkali reserve of the blood was also found in some of the thyroid-fed rabbits, the aerated blood dialysate having a reaction of pH 7.9 to 8.0. When greens were given freely to the thyroid-fed rabbits, such changes in the blood reaction could not be observed. In the control animals, either fasted or fed with animal tissue powder, the hydrogen ion concentration of the blood remained almost unchanged. The kind of food eaten leads to a marked difference in the CO_2 capacity of the blood plasma of the normal rabbits. When greens only were given, the plasma CO_2 capacity was 61.4 to 63.6 (cc. of CO_2, reduced to 0°C., 760 mm. Hg, bound as bicarbonate in 100 cc. of the blood plasma), while this value was reduced to 48.2 to 53.2 on oat diet. When thyroid was given to the animals, the CO_2 capacity of the plasma gradually decreased. On oat diet it went down as far as 28.7

Digitized by Google

TABLE II.

The Influence of Fasting or Feeding Animal Tissue Powder upon the Hydrogen Ion Concentration of the Blood and the Alkali Reserve of the Blood Plasma.

Experimental day.	Body weight.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit I. Fasting. (Before experiment oats only were given freely for 5 days.)						
	kg.				cc.	
1	2.04	7.45	8.1	50.1	60	Urine: protein −, casts −.
2		7.45	8.1	44.5	73	
3					50	
4		7.45	8.1	44.7	48	
5		7.45	8.1	41.9	63	Urine: protein ±, casts −.
6		7.40	8.1	36.0	79	Died on 7th day.
Rabbit II. Fasting. (Before experiment oats and greens were given freely for 5 days.)						
1	2.20	7.5	8.2	62.5	85	Urine: protein −, casts −.
2					133	
3		7.35	8.1	40.0	172	
4					?	
5		7.35	8.1	39.5	150	Urine: protein −, casts −.
6		7.35	8.1	34.7	136	
7		7.35	8.1	34.8	53	
8	1.38					Died.
Rabbit III. Animal tissue powder feeding, 3 gm. per day. No other food. (Before experiment oats only were given freely for 16 days.)						
1	1.78	7.45	8.1	48.2	55	Urine: protein −, casts −.
2					89	
3		7.5	8.1	52.9	153	
4					100	
5		7.4	8.1	44.3	65	
6					73	
7		7.4	8.1	41.3	93	
8					62	Urine: protein −, casts −.
9	1.22	7.4	8.1	42.0		Recovered by giving food.

Digitized by Google

TABLE II—*Concluded.*

Experimental day.	Body weight.	Blood. pH Before aeration.	Blood. pH After aeration.	Blood. Plasma CO_2 capacity.	Urine volume.	Remarks.
Rabbit IV. Animal tissue powder feeding, 3 gm. per day. No other food. (Before experiment oats and greens were given freely for 7 days.)						
	kg.				*cc.*	
1	1.70	7.4	8.1	55.3	62	Urine: protein −, casts −.
2					55	
3		7.4	8.1	45.0	70	
4					48	
5		7.4	8.1	40.9	59	Urine: protein +, casts −.
6					61	
7	1.40	7.4	8.1	38.5	45	Died 2 days later.

to 30.3, while on green diet the minimum was 43.8 to 46.2. In one case of thyroid-fed rabbits on the oat diet, thyroid feeding was stopped and greens were freely given after the 6th experimental day. The CO_2 capacity of the plasma increased from 29.6 to 60.7 in 2 days. In rabbits either fasted or fed with animal tissue powder, the CO_2 capacity of the plasma was also decreased, the former showing a more marked change than the latter. The minimum of the CO_2 capacity of the plasma was 36.0 to 34.7 (fasted) and 38.5 to 41.3 (animal tissue powder fed).

Judging from these results, it will be seen that in rabbits fed oats, thyroid feeding continued for several days caused a marked degree of acidosis, determined by both hydrogen ion concentration of the blood and the CO_2 capacity of the plasma, this disturbance, however, being reparable to a certain degree by taking a large enough amount of base-forming diet; and that the acidosis of thyroid-fed rabbits is greater than that of fasted or animal tissue powder-fed rabbits. Goto[11] reported that acidosis exists in uranium nephritis. In some of my rabbits the kidneys seemed to be slightly impaired. As this change, if existing at all, was very slight, acidosis observed in my experiments is probably not due to the kidney injury. Acidosis in diabetes is reported to

[11] Goto, K., *J. Exp. Med.*, 1917, xxv, 693.

Digitized by Google

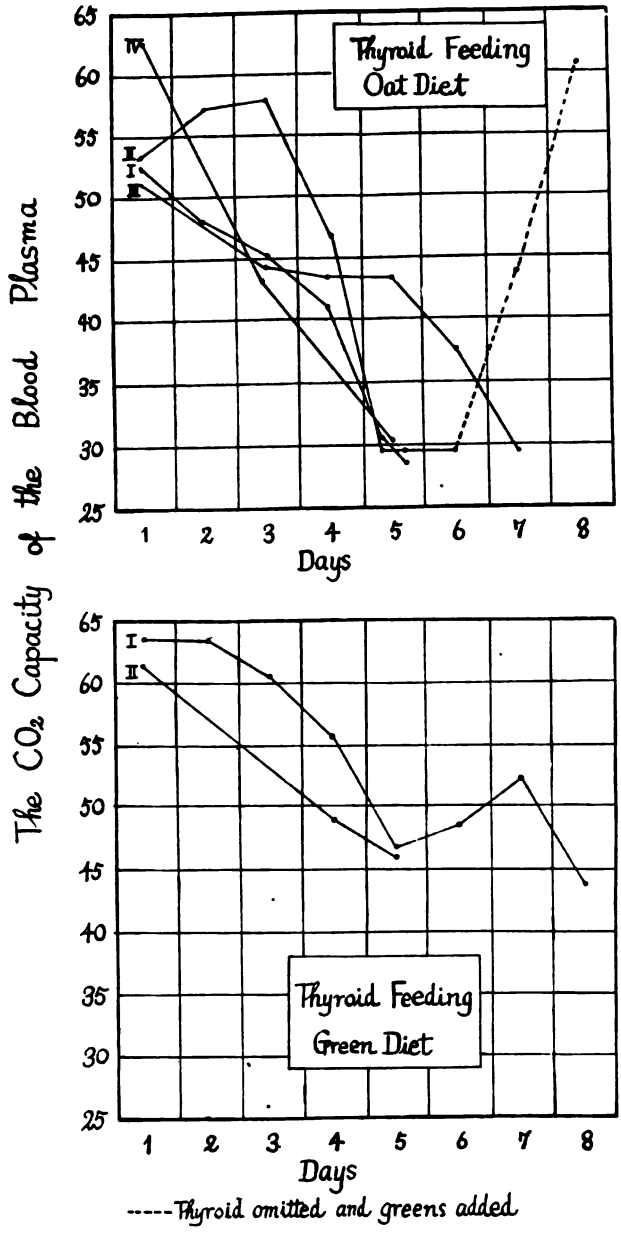


CHART 1. The influence of thyroid feeding upon the CO_2 capacity of the blood plasma.

Digitized by Google

be due to an overwhelmingly rapid production of acids, and acidosis in nephritis to an inability to eliminate acids which are produced at a moderate rate. Acidosis found in thyroid-fed rabbits is probably due to an abnormal increase of acid production, a shortage of base-forming diet accelerating this acidosis markedly.

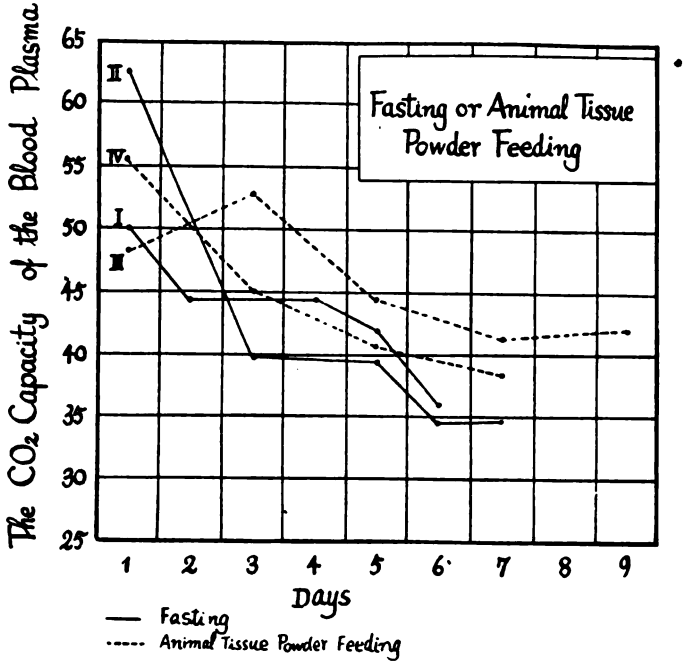


CHART 2. The influence of fasting or of feeding animal tissue powder upon the CO_2 capacity of the blood plasma.

Relations between Epinephrine in the Blood and Acidosis of Thyroid-Fed Rabbits.

Recently Peters and Geyelin[12] reported that subcutaneous injection of epinephrine causes a marked decrease of alkali reserve of the blood plasma, in either diabetic or normal men. This change was parallel with the epinephrine hyperglycemia, the maximum of both being obtained within 3 hours. They considered that at least a part of the hyperglycemia and glycosuria following the injection of epinephrine seems to be caused by a diminution of the alkalinity of the blood.

[12] Peters, J. P., and Geyelin, H. R., *J. Biol. Chem.*, 1917, xxxi, 471.

Digitized by Google

In order to see whether a diminution of the alkali reserve of the blood after thyroid feeding has any relation to epinephrine, Peters and Geyelin's experiments were repeated on normal rabbits.

Methods.—Adrenalin (1: 1,000 Parke, Davis and Company) was injected subcutaneously into full grown rabbits in doses of 0.5 gm. or 1 mg. of epinephrine per kilo of body weight. Before the experiments some rabbits were fed on oats only, the others on oats and greens for 4 days. Directly before epinephrine injection the animals were fasted for 24 hours. After epinephrine injection the CO_2 capacity of the plasma was determined as described in the preceding chapter. The urine in 24 hours after the injection was collected and its sugar content was determined polariscopically. The details are shown in Table III.

TABLE III.

The Influence of Epinephrine Injection upon the Alkali Reserve of the Blood Plasma. Food in Preparatory Period (4 Days), Nos. III and VI Oats Only; for Others, Oats and Greens.*

Rabbit			I†	II	III	IV	V	VI	VII
Body weight, *kg*			2.38	1.88	1.70	1.30	1.94	1.70	1.72
Plasma CO_2 capacity. (Cc. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 cc. of plasma.)	Before injection.		54.8	63.2	53.8	53.9	58.6	48.5	56.7
	Hrs. after injection.	½				59.5	56.7	50.7	59.5
		1				59.6	55.7	49.2	59.5
		2	61.9	55.6	47.2	60.0			
		4	62.4	59.5	54.4	63.3			
		7	52.8	58.6	54.5				
Urine in 24 hrs. after injection.	Volume, *cc.*		137	103	95	129	116	180	203
	Dextrose, *gm.*		0.81	1.47	0.56	0.42	0.40	0.39	1.79

* Epinephrine injection, 0.5 mg. per kilo of body weight in Rabbits I to VI; 1.0 mg. in No. VII.

† The sugar content of the blood before epinephrine injection, and 2, 4, and 7 hrs. after epinephrine injection respectively was 0.13, 0.33, 0.25, and 0.15 per cent.

Digitized by Google

From this table it will be seen that in normal rabbits epinephrine injection does not cause any noteworthy decrease of the CO_2 capacity of the plasma. Judging from the glycosuria and hyperglycemia, the drug employed was very effective. The sugar estimation in one case and my previous experiments[13] concerning the epinephrine hyperglycemia show that the maximal point of the epinephrine hyperglycemia is usually obtained from 2 to 4 hours after the subcutaneous injection of epinephrine. No marked decrease of the alkali reserve was observed even at this point. So far as shown by these experiments, no evidence was obtained that hypersecretion of the adrenals plays any rôle in acidosis found in thyroid-fed animals.

The Influence of Sodium Bicarbonate upon the Decrease of the Liver Glycogen of Thyroid-Fed Rats.

Pavy and Bywaters[14] reported that the postmortem production of sugar in the liver may be virtually checked by the injection of a·2 per cent solution of sodium carbonate into the portal system of the living animal. A similar effect of alkali upon the sugar production of the liver was reported by Pavy and Godden[15] in chloroform glycosuria, which is dependent upon the glycogen supply of the liver. A cat was made glycosuric by chloroform anesthesia, and then sodium carbonate, in 3 per cent solution, to the amount of 0.75 gm. per kilo of body weight, was injected into the femoral vein. This brought about a speedy reduction in the glycosuria. Murlin and Kramer[16] reported that in pancreatic diabetes, sodium carbonate decreases glycosuria and that glucose thus retained is not held back as glycogen. According to them, sodium bicarbonate had not the same effect as sodium carbonate. Underhill,[17] however, observed a very favorable effect of sodium bicarbonate in decreasing glycosuria in diabetic patients. Feeding normal rabbits on either base-forming diet (carrot) or acid-forming diet (grain), McDanell and Underhill[18] reported that the former is somewhat more efficient in the formation of liver glycogen than the latter.

In connection with the rapid decrease of the liver glycogen and an onset of acidosis in the thyroid-fed animals, I have made

[13] Kuriyama, S., *J. Biol. Chem.*, 1917, xxix, 127.

[14] Pavy, F. W., and Bywaters, H. W., *J. Physiol.*, 1910–11, xli, 168.

[15] Pavy, F. W., and Godden, W., *J. Physiol.*, 1911–12, xliii, p. vii.

[16] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1916, xxvii, 481. Kramer, B., Marker, J., and Murlin, J. R., *ibid.*, 1916, xxvii, 499.

[17] Underhill, F. P., *J. Am. Med. Assn.*, 1917, lxviii, 497.

[18] McDanell and Underhill, *J. Biol. Chem.*, 1917, xxix, 255.

Digitized by Google

some experiments on the influence of alkali upon the decrease of the liver glycogen of thyroid-fed animals.

Methods.—Desiccated thyroid was given to albino rats in doses of 1 gm. per day for 4 days. As the main food, dog biscuit-lard paste was used. 0.3 to 0.4 gm. of sodium bicarbonate was mixed with the food every day. When a larger amount of bicarbonate was given, the animal refused to eat it. On the 4th day 1 or 2 gm. of sucrose was given in some cases. In the morning of the 5th day, the liver glycogen was determined. The results are shown in Table IV.

TABLE IV.

The Influence of Sodium Bicarbonate upon the Decrease of Liver Glycogen of Thyroid-Fed Rats.

Rat			I	II	III	IV	V
Body weight, *gm*			255.7	218.6	217.4	209.6	230.1
Desiccated thyroid 1 gm. +	1st day.	Paste, *gm*.	8.0	6.9	6.8	6.6	4.5
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
	2nd day.	Paste, *gm*.	6.9	6.6	7.0	6.0	6.0
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
	3rd day.	Paste, *gm*.	6.8	6.0	6.2	6.6	6.0
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
	4th day.	Paste, *gm*.	6.9	6.8	6.9	6.4	8.0
		Sucrose, *gm*.	0	1.0	0	2.0	0
		$NaHCO_3$, *gm*.	0.3	0.3	0.4	0.4	0.4
		Calories.	38.6	42.2	38.6	44.3	44.1
Loss of body weight, *gm*			−8.8	−25.8	−24.0	−21.5	−21.5
Liver.	Weight, *gm*		9.4	6.8	6.3	5.7	6.8
	Glycogen content.	*mg*	0	10.9	Trace.	23.4	0
		per cent.		0.2		0.4	

From this table it will be seen that under the experimental conditions employed, an oral administration of a relatively small amount of sodium bicarbonate does not specially modify the de-

Digitized by Google

crease of the liver glycogen of thyroid-fed rats. The kind of alkali, the method of administration, and the dose must, however, be taken into consideration. Though it may be possible that the acidosis in experimental hyperthyroidism plays some rôle in restraining the new formation of liver glycogen, further investigations are needed for making this relation clear.

SUMMARY.

In normal rabbits the level of the alkali reserve of the blood is markedly dependent upon the character of the food. Though the CO_2 capacity of the plasma is 61 to 64 in green diet, this value is reduced to 48 to 53 on the oat diet. The hydrogen ion concentration of the blood remains constant with both kinds of diet (pH 7.35 to 7.5). Thyroid feeding induces a noteworthy acidosis in oat-fed rabbits (CO_2 capacity of the plasma, 29 to 30; pH of the blood, 7.25 to 7.3 before aeration, 7.9 to 8.0 after aeration). On green diet these changes are not so marked, probably being repaired by the base-forming character of the food (CO_2 capacity of the plasma, 44 to 46; pH of the blood, 7.45). The acidosis induced by thyroid in oat-fed rabbits can be promptly removed by omitting thyroid from the diet and adding greens. Acidosis appears also in rabbits either fasted or fed with animal tissue powder, but to a smaller degree than in the thyroid-fed animals (CO_2 capacity of the plasma, 36 to 35, and 41 to 39 respectively). Acidosis induced by thyroid feeding is probably due to an increased production of acids in the body.

Epinephrine injection into rabbits failed to cause any noteworthy acidosis. No evidence was obtained that the acidosis found in thyroid-fed rabbits is induced by hypersecretion of the adrenals.

When a small amount of sodium bicarbonate was orally administered into thyroid-fed rats, the decrease of the glycogen content of the liver was nearly as great as that in those without any excess alkali.

The author desires to express his thanks to Professor Frank P. Underhill for his suggestions and interest throughout the course of this and the two foregoing investigations; also to Professor Lafayette B. Mendel for his advice.

Digitized by Google

Digitized by Google

THE STRUCTURE OF YEAST NUCLEIC ACID.

II. URIDINEPHOSPHORIC ACID.

By P. A. LEVENE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATE 1.

(Received for publication, December 1, 1917.)

In a series of articles published in the course of the last few years, Walter Jones[1] and his coworkers advanced a theory on the mode of linkage of the four nucleotides taking part in the molecular structure of yeast nucleic acid. According to these authors, the nucleus of yeast nucleic acid is a tetra-ribose of the following structure $[(C_5H_{10}O_5)_4 - 3\ H_2O]$. Jones and his coworkers have based their conclusions on the analysis of three substances which they had regarded as dinucleotides.

In a previous publication[2] it was pointed out that the theory of Jones and his coworkers was not the only possible conclusion from the facts presented by them. It was further pointed out that not sufficient rigor had been exercised in proving the dinucleotide structure of the substances described by them. In the same publication the present author reported in a preliminary way the results of his own attempts to fractionate the pyrimidine nucleotides described by Levene and Jacobs.[3] For this purpose the crude nucleotides were transformed into the brucine salts and these were repeatedly recrystallized from dry methyl alcohol. In this manner a substance of constant composition was obtained. On conversion of the brucine salt into a barium salt, the composition of the substance was not altered. Both the brucine and the barium salt had the elementary composition of a di-

[1] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *ibid.*, 1916, xxv, 93. Jones, W., and Read, B. E., *ibid.*, 1917, xxix, 123; xxvi, 39.

[2] Levene, P. A., *J. Biol. Chem.*, 1917, xxxi, 591.

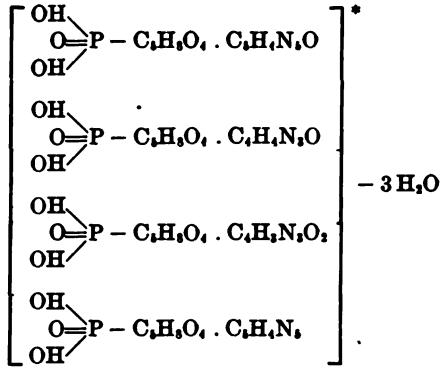
[3] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1911, xliv, 1027; *J. Biol. Chem.*, 1912, xii, 411.

Digitized by Google

nucleotide. And yet, such a conclusion did not appear entirely compelling, and further attempts at fractionation seemed desirable. Crystallization of the same crude brucine salts from a 35 per cent solution of ethyl alcohol was now resorted to. Passing the salts through eight recrystallizations, it was possible to separate them into two principal fractions. The less soluble part had a composition of the salt of uridinephosphoric acid and the more soluble that of cytidinephosphoric acid. Each of these could be converted into its barium salt. The barium salt of the uridinephosphoric acid precipitated out of a concentrated aqueous solution in the form of long needles, aggregated in rosettes resembling in form those of an osazone. On the other hand, the barium salt of the cytidinephosphoric acid appeared under the same conditions in the form of microscopic granules. The two salts differed in their solubility and in their optical rotation. In this communication only the results of the analysis of uridinephosphoric acid will be reported since attempts are still in progress to obtain the barium salt of the cytidinephosphoric acid also in crystalline form. The purity of the uridinephosphoric acid has been proven by the fact that amino nitrogen could not be detected on the analysis, either of the barium salt directly, or after previous hydrolysis. Nor was it possible to isolate from the product of its hydrolysis any other base than uracil. The specific rotation of the substance was $[\alpha]_D^{20} = +3.5°$.

Thus it is evident that the substance previously described as pyrimidine dinucleotide was a mixture of two mononucleotides. This observation is important inasmuch as it demonstrates conclusively that for the present there is no experimental proof for the assumption of a tetra-ribose as the nucleus of yeast nucleic acid. This, however, does not exclude the possibility that such proof may be furnished in the future. Meanwhile the structural formula of yeast nucleic acid, free from all arbitrary elements, may be written in the following form.

Digitized by Google



* Bottomley has recently (*Proc. Roy. Soc., B*, 1917, xc, 39) criticised the view expressed by Levene and Medigreceanu (*J. Biol. Chem.*, 1911, ix, 375, 389) that the first phase in the enzymatic cleavage of nucleic acid consisted in the dissolution of the union between individual mononucleotides; Bottomley modified the theory, accepting that the first phase of decomposition of nucleic acid is limited to its cleavage into two dinucleotides. This hypothesis may eventually prove correct; however, the evidence furnished by Bottomley is unsatisfactory. The author made no attempt to fractionate his crude material. For the present the conclusion of Bottomley seems to us unsustained by facts.

EXPERIMENTAL.

The condition of hydrolysis and the method of preparation of the mixed pyrimidine nucleotides were the same as previously described. Care was taken to keep the temperature of the oil bath during the hydrolysis at 100°C.

The crude silver salts were suspended in water and freed from silver by means of hydrogen sulfide. The filtrate from silver sulfide was freed from hydrogen sulfide by aeration, then rendered alkaline by means of a solution of barium hydroxide in order to remove the phosphoric acid. The filtrate was then neutralized and concentrated at diminished pressure and at 50°C. From the concentrated solution the barium was removed quantitatively and to the filtrate brucine in methyl alcoholic solution was added until the originally acid solution turned slightly alkaline to litmus. On standing, a crystalline deposit of the nucleotides formed.

The separation of the two nucleotides was brought about by recrystallization from 35 per cent ethyl alcohol. After eight recrystallizations the brucine salt of a pure uridinenucleotide was

Digitized by Google

obtained. In the purification of the brucine salt one may, to some extent, be guided by its melting point. The pure material on heating in a capillary tube first contracts at 183°C. (corrected), then melts, and finally decomposes at 198°C. (corrected). However, even after this phase is attained it is advisable to repeat recrystallization at least three times. The optical rotation of the substance was not measured because of its great insolubility in water and in other solvents. The solubility of the salt is greater in dilute alcohol than in water, but the solubility even in this reagent is not great enough to permit an accurate optical measurement.

The composition of the brucine salt (No. 70) was the following:

0.1027 gm. substance gave 0.1998 gm. CO_2 and 0.0584 gm. H_2O.
0.2000 " " " 12.0 cc. of nitrogen at t° = 22°C. and p = 764 mm.
0.2000 " " " 0.0168 gm. $Mg_2P_2O_7$.

	Calculated for $C_{28}H_{36}N_4PO_7 + 7\ H_2O$:	Found:
C	53.20	53.06
H	6.51	6.37
N	6.80	6.96
P	2.52	2.37

Conversion of the Brucine Salt into the Barium Salt.—The brucine salt was dissolved in hot water by means of an excess of ammonia water, and the brucine was extracted by means of chloroform in a separating funnel. The aqueous solution of the nucleotide was repeatedly evaporated to dryness under diminished pressure with an excess of barium hydroxide until all ammonium was removed. The residue was then dissolved by the aid of some sulfuric acid, and to the solution a barium hydroxide solution was added until the reaction turned slightly alkaline to phenolphthalein. The filtrate from the barium hydroxide was concentrated under diminished pressure at a temperature of the water bath not exceeding 50°C. It is preferable to interrupt the distillation just before the nucleotide begins to settle out while the distillation is still in progress, since in such case it may have an amorphous appearance. If desired the barium salt may be recrystallized out of water. When air-dry the substance has a granular appearance, and microscopically it consists of rosettes composed of long needles, as is shown in the figures.

Digitized by Google

Sample 57.

0.1024 gm. substance gave 0.0888 gm. CO_2, 0.0242 gm. H_2O, and 0.0502 gm. $Ba_2P_2O_7$.

0.1015 gm. used for Kjeldahl nitrogen estimation required for neutralization 4.47 cc. 0.1 N acid.

Sample 55.

0.1422 gm. substance used for Kjeldahl nitrogen estimation required for neutralization 6.36 cc. 0.1 N acid.

0.1422 gm. substance gave on fusion 0.0348 gm. $Mg_2P_2O_7$.

0.0711 " " " 0.0356 gm. $BaSO_4$.

	Calculated for $C_9H_{11}N_2O_8PBa$:	Found: No. 55	No. 57
C	23.50		23.65
H	2.41		2.64
N	6.10	6.27	6.16
P	6.75	6.82	
Ba	29.90	29.47	
$Ba_2P_2O_7$	48.97		49.02

The specific rotation of the Ba salt in a 2.5 per cent solution of HCl was

$$[\alpha]_D^{20} = \frac{+0.14 \times 100}{1 \times 4} = +3.5°$$

Hydrolysis of Barium Salt.—9.0 gm. of the barium salt were dissolved in 100.0 cc. of water to which 13.0 gm. of sulfuric acid were added and the solution was heated for 4 hours at 125°C. in a sealed tube. The sulfuric acid was removed quantitatively and the solution was concentrated under diminished pressure until uracil began to crystallize. The mother liquor from uracil was tested for amino nitrogen with a negative result. Also the attempt to prepare an insoluble picrate of cytosine was unsuccessful.

The uracil was recrystallized from dilute sulfuric acid and had the following composition:

0.0984 gm. substance was employed for a Kjeldahl nitrogen estimation and required for neutralization 17.49 cc. of 0.1 N acid.

	Calculated for $C_4H_4N_2O_2$:	Found:
N	25.05	24.88

Cytidinephosphoric Acid.—The mother liquor from the brucine salt of the uridinephosphoric acid was concentrated until a second

Digitized by Google

crystallization began to deposit. This second brucine salt was converted into a barium salt, which had the composition of the salt of cytidinephosphoric acid. The salt deposited in form of microscopic globules. Attempts are being made to obtain it in a crystalline form and, because of this, the results of the analysis of the substance are deferred to a later date.

Digitized by Google

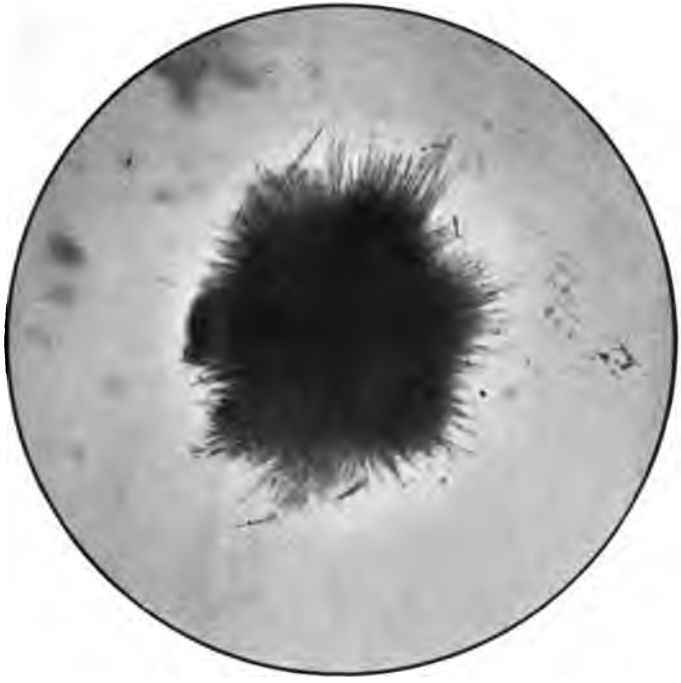


FIG. 1.

Digitized by Google

FIG. 2.

(Levene: Yeast nucleic acid.)

Digitized by Google

THE DETERMINATION OF SODIUM AND POTASSIUM.

By F. H. McCRUDDÉN and C. S. SARGENT.

(*From the Robert B. Brigham Hospital, Boston.*)

(Received for publication, November 26, 1917.)

Sodium and potassium can be separated from other compounds as NaCl + KCl, and the amount of each calculated from the chlorine content of a known weight of the mixture.

$$\begin{aligned} \text{NaCl} + \quad & \text{KCl} = \text{S}^1 \\ \tfrac{35.45}{58.50}\,\text{NaCl} + \tfrac{35.45}{74.56}\,\text{KCl} &= \text{H}^2 \end{aligned}$$

Though the possibilities of error are great, the method is, nevertheless, practicable.

The percentage error in NaCl: percentage error in Cl =

$$\frac{100\,\dfrac{\Delta\text{NaCl}}{\text{NaCl}}}{100\,\dfrac{\Delta\text{H}}{\text{H}}} = \frac{\Delta\text{NaCl}\,.\,\text{H}}{\Delta\text{H}\,.\,\text{NaCl}}$$

(ΔNaCl and ΔH being the absolute errors).

Solving the first two equations:

$$\text{NaCl} = \frac{\text{H} - \frac{35.45}{74.56}\text{S}}{\frac{35.45}{58.50} - \frac{35.45}{74.56}}$$

Differentiating NaCl with respect to H:

$$\frac{\delta\text{NaCl}}{\delta\text{H}} = \frac{1}{\frac{35.45}{58.50} - \frac{35.45}{74.56}}$$

Substituting these values, we get as percentage error in sodium

[1] Sum.

[2] Halide.

Digitized by Google

$$\frac{1}{\frac{35.45}{58.50}-\frac{35.45}{74.56}} \times \frac{H}{H-\frac{35.45}{74.56}S} \text{ or } \frac{H}{H-\frac{35.45}{74.56}S}$$

times that in chlorine,[3] a value which increases as the proportion of sodium in the mixture diminishes.

Magnification of Percentage Error in Chlorine $\left(\frac{H}{H-\frac{35.45}{74.56}S}\right)$.

NaCl : KCl	9:1	8:2	7:3	6:4	5:5	4:6	3:7	2:8	1:9
Magnification	5.0	5.3	6.1	7.0	8.1	10.0	13.0	18.4	35.8

The very small error in the McLean-Van Slyke chloride method[4] can abide such ten- or twenty-folding.

The steps in the McLean-Van Slyke method are as follows: (1) add standard silver nitrate to solution of chlorides; (2) make to definite volume, filter, take aliquot part of filtrate; (3) titrate excess of silver nitrate in aliquot part with standard potassium iodide.

The *error in measuring the silver nitrate* with a pipette[5] (experimentally determined) is 0.005 cc. in 20 cc., — 0.025 per cent. (We weighed the irregular amounts of silver nitrate used in our work; the use of a burette at this point would multiply several times the error shown in Table I.)

If no more than 5 per cent excess of silver nitrate is added, the *error in "definite volume and aliquot part"* will be only 5 per cent of the actual error in the measurements (5 per cent of 0.10 per cent = 0.005 per cent).

[3] The total chlorides (NaCl + KCl) can be weighed accurately to 0.1 mg.; but if any error is introduced at this point it will be multiplied

$$\frac{\frac{35.45}{74.56}S}{H-\frac{35.45}{74.56}S}$$

times in the final result.

[4] McLean, F. C., and Van Slyke, D. D., *J. Am. Chem. Soc.*, 1915, xxxvii, 1128.

[5] We used 0.1 N $AgNO_3$ and 0.02 N KI (approximately).

Digitized by Google

Titration Error.

Experiment No.	Weight of approximately 0.1 N $AgNO_3$.	Approximately 0.02 N KI solution.	KI used for each gm. $AgNO_3$ solution.	Deviation from the mean.
	gm.	cc.	cc.	
1	1.0462	5.75	5.495	0.032
2	1.0492	5.79	5.519	0.008
3	1.0482	5.78	5.514	0.013
4	1.0482	5.79	5.524	0.003
5	1.0489	5.80	5.530	0.003
6	1.0486	5.79	5.522	0.005
7	1.0473	5.82	5.557	0.030
8	1.0473	5.81	5.552	0.025
Average			5.527	0.015

The average deviation from the mean was 0.015 cc.; the extreme deviation only 0.03 cc. If we take 0.04 cc.—the weight of one drop of potassium iodide solution (average of six determinations)—as the titration error, the corresponding error in the (one-fifth as strong[5]) silver nitrate will be (one-fifth of 0.04 cc. =) 0.008 cc. or 0.04 per cent (of 20 cc.).[6]

The error in sodium (0.2 to 0.8 per cent) calculated from a 0.04 per cent error in chlorine is reasonably close to that actually found by experiment (Table I and Chart 1).

Only when five place logarithms are used is the computation error[7] safely less than the analytical error[8] (Table II, Chart 2).

5.8500 gm. of sodium chloride dissolved in water and made up to 1 liter weighed 1,004.1 gm.; 1 gm. of the solution contained 0.0058261 gm. of sodium chloride. 7.4600 gm. potassium chloride dissolved in water and made up to 1 liter weighed 1,005.2 gm.; 1 gm. of the solution contained 0.0074215 gm. of potassium chloride.[9]

[6] Nothing is gained by making the potassium iodide solution either stronger or weaker; with a stronger solution the error is still about one drop—an increase in the absolute error. With a weaker solution, the absolute error increases proportionately; the proportional error remains the same.

[7] The error coming from not preserving enough significant figures in the calculations.

[8] "Four place tables should not be employed upon work of an accuracy exceeding one-half of one per cent," Holman, Computation Rules and Logarithms, New York, 1913, p. lxxvi.

[9] The pure chlorides were made from the purest obtainable commercial products by twice precipitating the salts from saturated aqueous solutions with hydrochloric acid gas. The quantities of these solutions used in all analyses were accurately weighed on the analytical balance.

Digitized by Google

Chlorine Equivalence of 1 Gm. of Silver Nitrate Solution.

	Against NaCl.	Against KCl.
1	0.0033749	0.0033773
2	0.0033746	0.0033773
3	0.0033754	0.0033746
Average....	0.0033750	0.0033764
Average....	0.0033757	

TABLE I.

Comparison of Calculated and Actual Error.

Parts.		Gm. used.		NaCl found.	Percentage error.		
NaCl	KCl	NaCl	KCl		Found.	Average.	Calculated.
		gm.	*gm.*	*gm.*			
8	2	0.10293	0.022813	0.10288	0.05		
		0.10239	0.023311	0.10233	0.06	0.09	0.21
		0.10386	0.023378	0.10370	0.15		
5	5	0.064012	0.067729	0.064155	0.22		
		0.064460	0.067476	0.064699	0.37	0.33	0.32
		0.064327	0.067907	0.064590	0.4		
4	6	0.053420	0.082394	0.053620	0.4	0.23	0.40
		0.052781	0.082571	0.052756	0.05		
3	7	0.041203	0.094061	0.040820	0.9	0.8	0.52
		0.041070	0.093326	0.040780	0.7		
2	8	0.029556	0.11191	0.030209	2.21		
		0.029288	0.11103	0.029673	1.31	1.9	0.74
		0.029487	0.11296	0.030147	2.24		

Digitized by Google

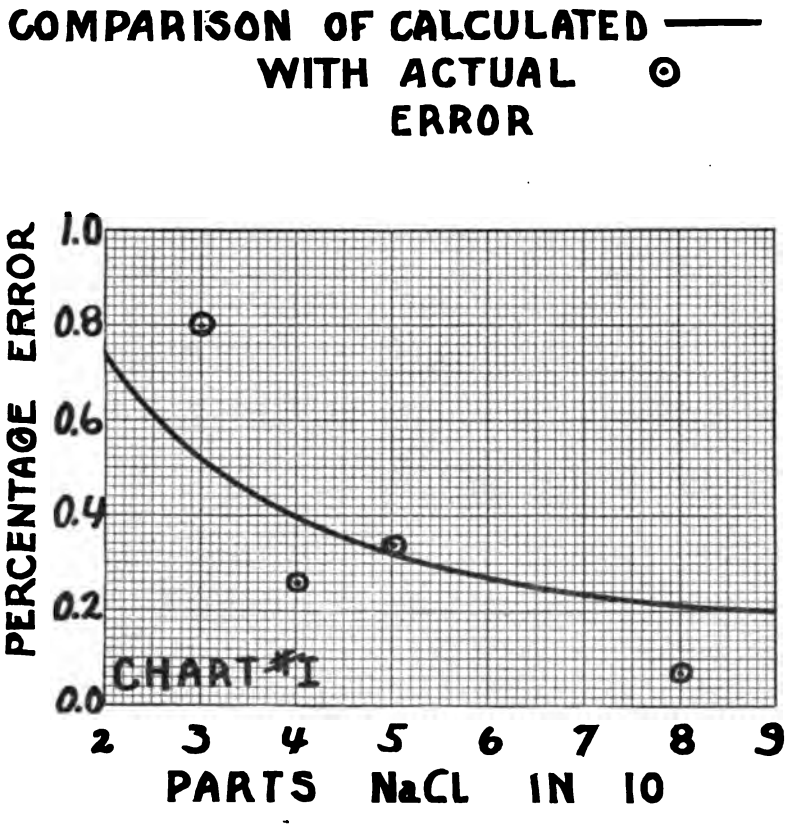


CHART 1. Calculated and actual error. Abscissæ = parts of NaCl in ten parts of NaCl + KCl. Ordinates = percentage error in sodium. Curve = calculated error in sodium for 0.04 per cent error in Cl.

Digitized by Google

TABLE II.

Comparison of Computation Errors.

Parts.		NaCl					Computation error.					
		Added.	Found.				Slide rule.		Logarithms.			
				Logarithms.					Four place.		Five place.	
NaCl	KCl		12 inch slide rule.	Four place.	Five place.	Six place.		Average.		Average.		Average.
		gm.	*gm.*	*gm.*	*gm.*	*gm.*						
8	2	0.102924	0.1030	0.1030	0.10288	0.102837	0.16		0.16		0.04	
		0.102338	0.1028	0.1025	0.10233	0.102263	0.54	0.3	0.24	0.14	0.07	0.06
		0.103862	0.1034	0.1036	0.10370	0.103623	0.29		0.02		0.07	
5	5	0.064012	0.0650	0.06412	0.064155		1.3		0.05			
		0.064460	0.0650	0.06459	0.064699		0.46	0.5	0.17	0.075		
		0.064327	0.06455	0.06459	0.064590		0.06		0.0			
4	6	0.053420	0.0528	0.05384	0.053620		1.5	0.8	0.4	0.28		
		0.052781	0.0528	0.05284	0.052756		0.1		0.16			
3	7	0.041203	0.0398	0.04076	0.04082		2.4	1.6	0.15	0.28		
		0.041070	0.0405	0.04061	0.04078		0.7		0.4			
2	8	0.0295559	0.03138	0.03038	0.030209	0.0302909	3.6		0.3		0.27	
		0.0292872	0.02984	0.02954	0.029673	0.0297518	0.3	1.7	0.7	0.58	0.27	0.27
		0.0294860	0.03059	0.03000	0.030147	0.0302259	1.2		0.75		0.27	

Digitized by Google

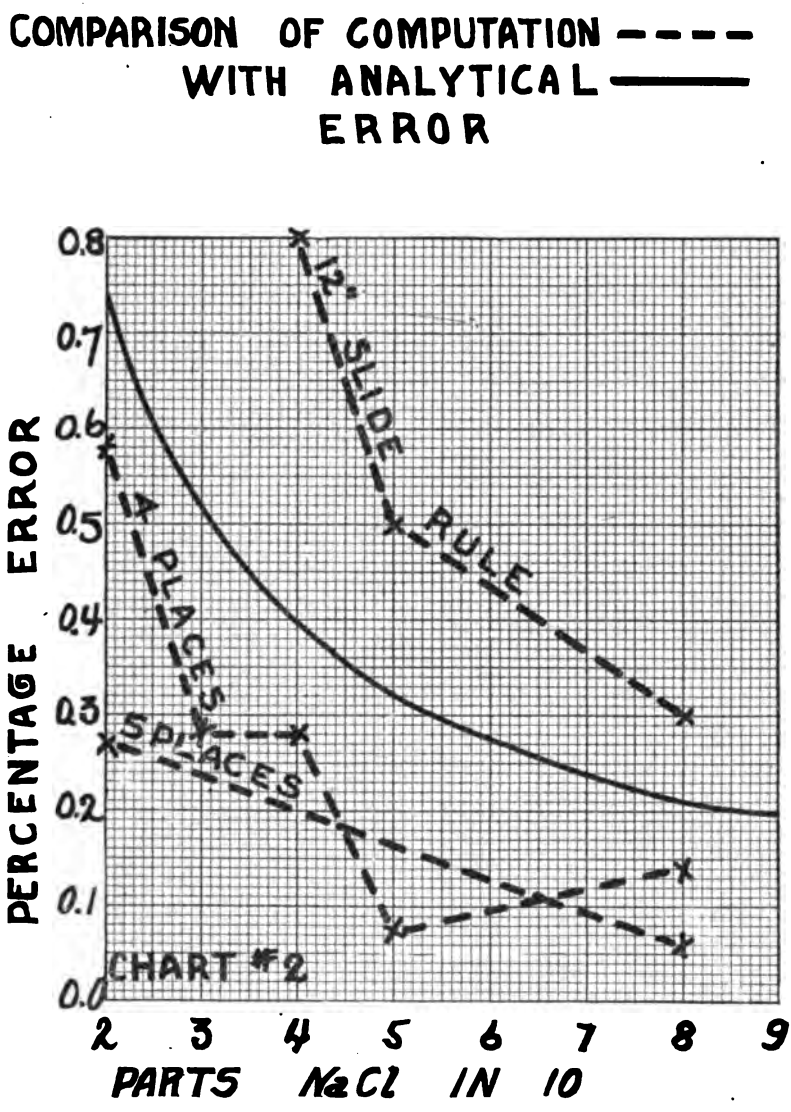


CHART 2. Computation error. Abscissæ = parts of NaCl in ten parts of NaCl + KCl. Ordinates = percentage error in sodium. Full line = analytical error. Dotted lines = computation errors.

Digitized by Google

Digitized by Google

SOME NEW CONSTITUENTS OF MILK.*

THIRD PAPER.

A NEW PROTEIN, SOLUBLE IN ALCOHOL.

BY THOMAS B. OSBORNE AND ALFRED J. WAKEMAN.

WITH THE COOPERATION OF CHARLES S. LEAVENWORTH AND OWEN L. NOLAN.

(*From the Laboratory of the Connecticut Agricultural Experiment Station, New Haven.*)

(Received for publication, December 6, 1917.)

From the residue obtained by concentrating the alcoholic washings from a large quantity of casein, which had been several times dissolved in dilute alkali and precipitated by dilute hydrochloric acid, we separated a not inconsiderable amount of protein which, like gliadin from wheat flour, was freely soluble in alcohol of 50 to 70 per cent by volume, but insoluble in absolute alcohol, and largely so in water containing more or less inorganic salts. The behavior of this protein towards solvents was so similar to that of gliadin and so unlike that of any protein of animal origin, hitherto described, that it seemed worth while to study it in considerable detail. That it is distinct from all of the other proteins heretofore obtained from milk was shown, not only by its behavior towards solvents and the proportion of some, at least, of the amino-acids which it yields on hydrolysis, but also by the fact that it does not react anaphylactically with any of these other proteins although it is itself highly anaphylactogenic. That this protein is an original constituent of milk and not a product of the action of alkali or other reagents used in purifying casein is shown by the fact that it can be obtained from casein which has been separated from milk simply by the addition of

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

Digitized by Google

hydrochloric acid. Owing to the fact that its acid compounds are soluble in water it can also be obtained from milk serum from which casein has been precipitated by acid and the coagulable proteins by heat.

From the purified casein which has been prepared in this laboratory we have obtained large volumes of dilute alcoholic extracts from which several hundred grams of this unique protein have been isolated in the following manner.

Casein was precipitated with dilute hydrochloric acid from centrifugated milk, filtered out, pressed, suspended in water, and dissolved, while actively stirring, by gradually adding dilute sodium hydroxide solution. From the resulting solution, which was but slightly alkaline to litmus, the casein was precipitated by dilute acetic acid. After twice reprecipitating in this way the casein was again suspended in water, dissolved by adding very dilute sodium hydroxide solution, precipitated by highly diluted hydrochloric acid, and filtered out on cheese-cloth. A sample of the filtrate when evaporated to dryness left a residue, chiefly sodium chloride, which dissolved completely in water, gave no biuret reaction, and no reduction with Fehling's solution.

The moist casein was then suspended in about an equal volume of 92 per cent alcohol, stirred for a short time, filtered out on cheese-cloth, and the extract filtered clear through paper pulp. In this way we obtained 43 liters of the first alcoholic extract, which was acid to litmus and contained 43 per cent by weight of alcohol and 350 gm. of solids, much of which was sodium chloride. The casein was again stirred up with more 92 per cent alcohol and 16 liters of a second extract were obtained containing 54 per cent of alcohol and 113 gm. of solids.

Of the first alcoholic extract 40 liters were concentrated to one-half, under diminished pressure, below 75°. The turbid concentrated extract on standing deposited on the bottom of the jars a transparent syrupy sediment, A. From this the nearly clear solution was decanted and concentrated to about 15 liters in open dishes. On cooling over night to room temperature a coherent deposit, B, formed.

A and B were dissolved in a little warm 70 per cent alcohol and the somewhat turbid solution was poured into several volumes of distilled water whereby an opaque colloidal "solution" was

Digitized by Google

produced. On adding a little sodium chloride a rapidly settling flocculent precipitate separated, leaving very little except salt in the nearly clear solution. On standing a short time the precipitate contracted to a coherent mass from which the solution was decanted almost completely.

When this precipitate was treated with about 600 cc. of absolute alcohol and warmed it yielded a solution from which a very little suspended flocculent substance was removed by filtering. The filtrate was then concentrated *in vacuo* until much protein separated in consequence of the loss of alcohol. On adding absolute alcohol and warming, a syrupy solution resulted which remained clear on cooling. This was poured into about 3 liters of absolute alcohol, whereupon most of the dissolved protein was precipitated. On adding a few drops of a strong solution of ammonium acetate the rest separated, leaving the solution clear. The precipitate was sucked out on a Buchner funnel, washed twice with absolute alcohol, and three times with absolute ether. When dried over sulfuric acid it formed a snow-white, friable mass which weighed 89 gm. and was easily reduced to a fine powder. This was designated preparation A.

Solution B, page 244, was evaporated to about 10 liters and cooled over night to room temperature. The solution was decanted from the coherent deposit which separated. The latter was rinsed with water, dissolved in warm 70 per cent alcohol, its solution filtered clear through paper pulp, and concentrated *in vacuo*, until much of the dissolved protein separated. Absolute alcohol was then added and the mixture warmed. A clear syrupy solution resulted, which remained clear on cooling. This was then poured into absolute alcohol and the protein, which separated almost completely, was sucked out, washed with absolute alcohol, and then with dry ether. When dried over sulfuric acid 46 gm. of preparation B were obtained.

The solution from which B had first separated was further concentrated to about 5 liters and, by the same procedure as employed for B, 28 gm. of preparation C were secured.

The 16 liters of the second alcoholic extract of the casein, page 244, were concentrated *in vacuo* to about 3 liters and cooled to room temperature. Nearly all of the dissolved protein separated as a coherent deposit. When subjected to the same treat-

Digitized by Google

ment as employed in making preparation B, 59 gm. of preparation D were obtained. Preparations A, B, and C represent successive fractional precipitations, while D includes all of the protein removed by extracting the casein a second time with somewhat stronger alcohol.

The following analyses show no appreciable differences between these several fractions of this peculiar protein.

Preparation	A	B	C	D
	per cent	*per cent*	*per cent*	*per cent*
Moisture at 110°	9.49	5.99	7.75	8.09
Ash	1.16	0.62	1.73	0.57
Calculated Ash- and Moisture-Free.				
	15.80	15.67	15.70	15.49
Nitrogen	15.87	15.67	15.70	15.67
		15.88		
Sulfur	0.97	0.94	0.94	1.00
Phosphorus	0.06	0.03	0.10	0.03

A composite sample of these four preparations was analyzed for carbon and hydrogen with the following results, calculated moisture- and ash-free.

Carbon	55.07	54.86
Hydrogen	7.24	7.23

A fifth preparation of this protein was made from the first and second alcoholic washings of another lot of casein which had been precipitated from about 500 liters of milk. The protein which separated after cooling the concentrated washings was dissolved in a little warm 70 per cent alcohol and its solution poured into about 50 liters of cold water. On adding sodium chloride most of the protein separated. The precipitate was collected on filters, redissolved in warm 70 per cent alcohol, and the solution filtered through paper pulp. The filtrate and washings (about 2 liters) were concentrated *in vacuo* to about 800 cc. with sufficiently frequent additions of strong alcohol to keep the protein dissolved. The concentrated solution was poured into a large volume of absolute alcohol and the resulting precipitate sucked out, washed first with absolute alcohol and then with ether. This preparation, E, when dried in warm air weighed 112 gm., and had the following composition.

Digitized by Google

	per cent	
Moisture	5.60	
Ash	1.12	

Ash- and Moisture-Free.

Carbon	54.81	
Hydrogen	7.06	
Nitrogen	15.71	15.69
Sulfur	0.89	
Phosphorus	0.28	

Comparison of the average of the above figures with the corresponding data for casein shows the alcohol-soluble protein to contain distinctly more carbon and sulfur and less phosphorus.

	Alcohol-soluble protein.	Casein.
	per cent	*per cent*
Carbon	54.91	53.11
Hydrogen	7.17	7.05
Nitrogen	15.71	15.65
Sulfur	0.95	0.82
Phosphorus	0.08	0.82
Oxygen (by difference)	21.18	22.55
	100.00	100.00

Determinations of the distribution of nitrogen, according to Hausmann's modified method, and also the proportion of basic amino-acids, according to Kossel are of interest since all known alcohol-soluble proteins of vegetable origin are characterized by a large proportion of amide nitrogen and a small proportion of basic nitrogen, especially of lysine.

The results obtained were as follows:

Partition of Nitrogen in the Alcohol-Soluble Milk Protein.

Preparation	A	B	C	D	Casein.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Amide nitrogen	1.65	1.56	1.55	1.51	1.62
	1.63	1.56	1.55	1.51	
Basic "	2.28	2.55	2.80	2.43	3.49
	2.48		2.87		
Humin "	0.26	0.21	0.24	0.22	0.21
	0.28		0.22		

Digitized by Google

Amino-Acids per 100 Gm. of Milk Protein.

	Alcohol-soluble protein.	Casein.
	gm.	gm.
Arginine	2.92	3.81
Histidine	2.28	2.50
Lysine	3.98	5.95
Tyrosine	2.47	4.50

These results show that casein yields much more basic nitrogen, as well as more arginine, histidine, or lysine, than does the alcohol-soluble milk protein and demonstrate that these proteins are distinctly different in structure.

The alcohol-soluble protein of milk does not yield more amide nitrogen, nor less basic nitrogen, or lysine, than do most other proteins, and in these respects does not resemble the alcohol-soluble proteins of vegetable origin, although so like wheat gliadin in its solubility in relatively strong alcohol.

Further evidence that this peculiar protein has no genetic relation to casein is shown by the results of anaphylaxis experiments made by Professor H. G. Wells with a preparation which we sent him, and also with a preparation of the casein from which the alcohol-soluble protein had been extracted by dilute alcohol.

Sensitizing Dose.		*Intoxicating Dose.*		*Result.*
	gm.		gm.	
Casein	0.001	Casein	0.05	Died in 15 min.
"	0.001	"	0.05	" " 5 "
Alcohol-soluble protein	0.001	Alcohol-soluble protein	0.05	" " 15 "
"	0.001	"	0.05	" " 10 "
"	0.001	Casein	0.05	No symptoms.
"	0.001	"	0.05	" "

Since acid compounds of the alcohol-soluble protein are soluble in water it is probable that a further quantity is present in the milk serum from which the casein had been precipitated. Examination of the alcoholic extract of large quantities of the coagulated protein obtained by boiling the acid filtrate from the casein failed to reveal the presence of any of this protein therein.

On the other hand some was found in the filtrate from the coagulated proteins. Thus 20 liters of milk were made neutral, first to litmus and then to phenolphthalein, and the precipitates were successively filtered out. Such neutralization precipitates contain considerable protein, but numerous attempts have failed to detect the presence of the alcohol-soluble protein in them.

Digitized by Google

The filtrate from the last precipitate was made neutral to litmus with hydrochloric acid and evaporated until a large part of the lactose was removed by successive crystallizations. The mother liquor (780 cc.) was saturated with ammonium sulfate, the precipitated proteins were dissolved in water, and an equal volume of saturated ammonium sulfate solution was added which precipitated much of the protein. This fraction was dissolved in water and alcohol added to 74 per cent by volume. The precipitate produced when dried over sulfuric acid weighed 2.3 gm., Fraction I.

The alcoholic filtrate from Fraction I was treated with an excess of strong alcohol and a little ammonium acetate solution added, which caused a practically complete precipitation of the rest of the protein. This was washed by centrifuging with 92 per cent alcohol and then centrifuged with alcohol of 70 per cent (by volume) in which much dissolved. After repeating this treatment three times a turbid, colloidal solution resulted. On adding five or six drops of a strong solution of ammonium acetate some protein was precipitated. This, Fraction II, was filtered out, and when dried over sulfuric acid weighed 0.55 gm. The clear brown filtrate (200 cc.) was poured into a liter of absolute alcohol, a few drops of ammonium acetate were added, and the flocculent precipitate was centrifuged out. When dried over sulfuric acid this fraction, No. III, weighed 1.8 gm.

To determine whether Fraction III which was soluble in relatively strong alcohol contained the alcohol-soluble protein the anaphylaxis reaction was used. The results obtained by H. G. Wells were as follows:

Sensitizing Dose.	*Intoxicating Dose.*	*Reaction.*
Alcohol-soluble protein.	Alcohol-soluble protein.	Died in 15 min.
" "	" "	" " 10 "
Fraction I.	" "	None.
" I.	" "	"
Alcohol-soluble protein.	Fraction I.	"
" "	" I.	"
Fraction III.	" I.	"
" III.	" I.	"
" III.	Alcohol-soluble protein.	Severe.
" III.	" "	Died in 5 min.

Digitized by Google

These experiments show that Fraction III sensitized guinea pigs to the alcohol-soluble protein obtained from the alcoholic washings of casein, but not to Fraction I. That no anaphylaxis reaction was obtained between Fractions I and III would at first sight seem strange, for while the methods employed for separating these fractions might lead to a fairly complete separation of the alcohol-soluble protein from Fraction I a complete separation of the latter from Fraction III could hardly be expected. The failure of Fractions I and III to react with one another, however, can be explained by the fact that numerous anaphylaxis experiments with many different preparations of the proteose-like products which we have obtained from milk showed that these were mostly destitute of anaphylactogenic power. The slight reactions which were occasionally obtained can now be easily explained by the presence of the alcohol-soluble protein in some of the preparations used.

Properties of the Alcohol-Soluble Milk Protein.

The preparations of the alcohol-soluble milk protein are unquestionably acid compounds, for when 1 gm. is suspended in 50 cc. of water 2.5 cc. of 0.1 N NaOH solution are required to obtain a neutral reaction to phenolphthalein. A slightly turbid solution is thus formed which becomes distinctly more turbid when 0.5 cc. of 0.1 N HCl is added. On continuing the addition of acid up to 1.3 cc. a coarse flocculent precipitate separates leaving the solution practically clear. At this reaction very little remains dissolved.

Conversely when 1 gm. is suspended in 50 cc. of water a turbid solution results in which a not inconsiderable quantity remains undissolved. When 0.1 N NaOH solution is gradually added the protein separates in coarse flocks, nearly all being precipitated when 1.2 cc. has been added; *i.e.*, at the same reaction as that at which it separates on adding acid to the solution neutralized to phenolphthalein. When an equal volume of absolute alcohol is added most of the suspended protein dissolves after vigorous stirring. On standing, the slightly turbid solution deposits a transparent varnish-like layer which is readily soluble in warm 70 per cent alcohol, but from which a part separates on cooling

Digitized by Google

to 20°. On adding water to this transparent deposit it becomes opaque and fails to dissolve. Possibly this less soluble fraction is an acid compound which differs from the more soluble part in the kind or quantity of combined acid. The solution in alcohol of 50 per cent by volume remains clear on adding alcohol to 70 per cent at about 20°. The solubility in alcohol of various degrees of concentration depends much on the temperature. Below 30° the solubility rapidly diminishes. When suspended in water containing a very little acetic acid the alcohol-soluble milk protein dissolves completely. This solution becomes opalescent when heated to about 45° and at 80° is milk-white, but no flocculent coagulum separates on boiling. When an equal volume of absolute alcohol is then added a water-clear solution results at once. When this clear solution is poured into water an abundant separation occurs. If, instead of alcohol, sodium chloride is added, no visible separation of particles takes place, but, on standing, the solution separates into strata of differing opacity and an oily deposit slowly forms which is readily soluble in relatively strong alcohol.

Dissolved in very dilute acetic acid this protein yields a voluminous precipitate on adding potassium ferrocyanide.

It gives strong tryptophane, Millon's, and biuret reactions.

From the behavior of this protein towards water, and its reaction with potassium ferrocyanide it is evident that it is not to be considered as belonging to any group of the proteoses. Its preeminent solubility in relatively strong alcoholic solutions distinguishes it from any type of protein heretofore discovered in animal tissues or products. The possible existence of proteins of similar solubility ought to be considered whenever the complete removal of protein is necessary for the isolation of non-protein nitrogenous substances of animal origin.

Digitized by Google

Digitized by Google

STUDIES IN THE METABOLIC CHANGES INDUCED BY ADMINISTRATION OF GUANIDINE BASES.

I. INFLUENCE OF INJECTED GUANIDINE HYDROCHLORIDE UPON BLOOD SUGAR CONTENT.

By C. K. WATANABE.

(From the Laboratory of Pathological Chemistry, School of Medicine, and the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

(Received for publication, December 13, 1917.)

It is indicated in the work of many former investigators that there exists an intimate relation between idiopathic tetany and disturbed parathyroid function. The fact that the symptoms of tetany and tetania parathyreopriva are almost identical has had experimental confirmation.

Paton and Findlay (1) confirmed the results of former workers in a series of critical investigations, in which they found that true tetany developed only after complete parathyroidectomy and that when one external parathyroid was left with its blood supply intact, no symptoms, or at most transient tremors, appear.

Many hypotheses concerning the cause of tetany have been proposed, especially from the standpoint of parathyreopriva. One states that the lack of the parathyroid produces a toxic substance in the body, or the normal function of this gland is to neutralize the toxins formed during normal metabolism. The other concerns the deficiency of some essential substance in the blood and tissues, especially calcium, which exerts an inhibition on the hyperexcitability of the nerves. It is well known that injection of calcium salts removes the symptoms for a time, but that bleeding and transfusion of saline solution (2, 3) and that bleeding followed by injection of an indifferent solution free from calcium (Ringer's solution) (4) have the same effect. These facts seem to point out that the Ca deficiency is not the true cause. Beebe's experiments in which he reduced the symptoms by injection of the extracts of parathyroids (5) and of the nucleoprotein of parathyroids (6), lead us to believe that the symptoms are due to some poison produced as a consequence of normal metabolism incident to the removal of the parathyroid. Recently Paton and Findlay (7) suggested that in tetany some toxic substances, especially guanidine

Digitized by Google

and methyl guanidine, exert a hyperexcitability on the nerves. Methyl guanidine was first isolated from normal human urine as the aurochloride by Kutscher and Lohmann (8) in 1906 and later by Engeland (9). Achelis (10) isolated it from normal dog urine as the picrolonate. The quantities found by the above three investigators correspond to about 0.007 gm. of the gold salt per liter. Koch (11) detected methyl guanidine in the urine of a dog which had been subjected to parathyroidectomy. In this case as much as 1.9 gm. per liter of the gold salt of the drug were found. In his second paper (12) he described more fully the occurrence of methyl guanidine and some of the other toxic bases in the urine in five parathyroidectomized dogs and stated that in all cases that he studied he found methyl guanidine. In those cases in which the quantity of methyl guanidine was small, other guanidine bases were present so that he considered the quantity of guanidine nitrogen to be constant in all cases. Burns and Sharpe (13), using more satisfactory methods than former workers, carefully determined the guanidine and methyl guanidine in the blood and urine of dogs after parathyroidectomy and reported a marked increase. They found an increased content of guanidine and methyl guanidine in the urine of children suffering from idiopathic tetany. They compared normal and abnormal dogs and showed an increase in guanidine of 90 per cent in blood and 40 per cent in the urine after parathyroidectomy. In the urine of children in condition of active tetany there was an increase of 500 per cent per liter over the normal. Such an increase of a spasmodic agent as guanidine suggests the cause of the development of the symptoms of tetany.

Paton and Findlay (7) demonstrated with careful observation on rabbits that the symptoms of tetania parathyreopriva are identical with those produced by the administration of the salts of guanidine and methyl guanidine.

In addition to the above workers, Underhill and others published a series of papers on carbohydrate metabolism in thyreoparathyroidectomized dogs. Hirsch (14), Eppinger, Falta, and Rudinger (15), and Pari (16) demonstrated that the assimilation limit for dextrose given by mouth or subcutaneously is lowered in thyreoparathyroidectomized dogs. Underhill and Saiki (17) also reported that thyreoparathyroidectomized dogs are incapable of utilizing subcutaneously introduced dextrose. They show a lessened oxidation or glycolysis and also a decreased ability to transform dextrose into glycogen. Underhill and Hilditch (18) observed that when the thyroids and all the parathyroids attached are removed from dogs, the ability to assimilate sugar is decreased. When the thyroid alone is extirpated this ability is not impaired. They consider that the thyroid-parathyroid mechanism stands in intimate connection with carbohydrate metabolism and that the parathyroids are the more active agents in this connection. Underhill and Blatherwick (19) found that during the tetany after thyreoparathyroidectomy, glycogen entirely disappeared from the liver and that the blood sugar content was markedly lowered. They attribute this phenomenon to the lack of parathyroid tissue since

Digitized by Google

this action may be observed only after entire parathyroidectomy and not when the removal of the thyroid leaves the parathyroids intact. Underhill and Blatherwick (20) also demonstrated that this hypoglycemia can be restored to normal temporarily and the tetany abolished for a time by the injection of calcium lactate.

From the work of Paton and Findlay (1) it appears that the symptoms of idiopathic tetany are identical with those of tetania parathyreopriva and that in both conditions there is an increased excretion of guanidine base (13) which is a well known convulsant toxin. The injection of such drugs induced the almost identical symptoms of tetania parathyreopriva (7). Therefore, it is possible that the chief cause of tetany is the production of relatively enormous quantities of guanidine bases in the body as a result of the impairment of the parathyroid function. Furthermore, if these guanidine bases are the sole source of the symptoms of tetany it seems interesting to inquire whether there is any correlation between the hypoglycemia produced by the parathyroidectomy and the guanidine content of the blood.

The present investigation deals with the influence of the injection of guanidine bases upon carbohydrate metabolism, especially the sugar content in the blood.

Methods.

Rabbits were used in this investigation. Blood was usually drawn from the ear vein before and after the subcutaneous injection of a 10 per cent solution of guanidine hydrochloride. McDanell's (21) modification of the Lewis-Benedict method was used for the estimation of sugar in the blood and Fehling's test for sugar in the urine. Duplicate determinations were done on the blood sugar to avoid technical errors. It was necessary to take some blood samples at night. In this case the specimen was immediately evaporated to dryness with the picric acid and determined colorimetrically the following morning. All specimens were evaporated to dryness with picric acid immediately on being drawn in order to avoid disappearance of sugar.

For the determination of total solids, the blood was drawn from the ear vein into a weighed crucible and dried to constant weight in an electric oven. About 2 cc. of blood were used for this determination and the sample for the blood sugar determination was taken at the same time. Comparison was made between normal rabbits and rabbits which had been injected with guanidine.

To determine the sublethal dose of guanidine hydrochloride

Digitized by Google

we used six rabbits and carefully observed the symptoms after the injection of various quantities of guanidine. The following table shows that 0.2 gm. per kilo of body weight is suitable for this purpose. As a rule all the rabbits which were used in the present work had neither food nor water for 24 hours after the injection. Immediately after the injection the rabbits usually

TABLE I.

Symptoms after Injecting Various Doses of Guanidine Hydrochloride.

Rabbit No.	Date.	Weight.	Guanidine per kilo.	Time of injection.	Symptoms and remarks.
	1917	*gm.*	*gm.*		
1	June 26	2,606	0.10	2.07 p.m.	No symptoms, recovered.
2	" 27	2,500	0.15	2.15 "	3.30 p.m. Dyspnea, twitching of head, jerking of head backwards and forwards, tremor in extremities, lying down with hind legs stretched. Symptoms disappeared by 5 p.m. Recovered.
3	" 28	2,700	0.20	2.23 "	No symptoms except slight dyspnea. Recovered.
4	" 28	2,500	0.25	10.50 a.m.	11.30 a.m. Lying down with hind legs stretched, dyspnea, chattering of teeth, jerking of head. Symptoms disappeared by 1 p.m. Died.
5	" 28	2,800	0.30	10.55 "	Quiet. No other symptoms. Died.
6	" 29	2,600	0.40	11 "	12.30 p.m. Lying down with hind legs stretched, sometimes twitching of extremities, especially hind legs. Paralysis appeared later in legs. Convulsions. Died.

run about because of the irritation produced by the injected drug. They remain quiet thereafter until the typical tetany symptoms appear—usually 1 to 1½ hours after the injection. In some cases in which small doses were administered the animal remained quiet and depressed; in other cases the animal became restless and showed slight dyspnea. When large doses were

Digitized by Google

administered the following symptoms appeared almost without exception, though usually not all in the same rabbit: dyspnea, depression, emaciation, twitching of head and legs, continuous jerking of head backwards and forwards, chattering of the teeth, fine tremor of the muscles, fibrillous tremor of extremities, opisthotonus, nystagmus following convulsion, extension of the hind legs, diarrhea, salivation, and disturbance of equilibrium.

Hypoglycemia after Injection of Guanidine Hydrochloride.

At first we administered 0.15 gm. per kilo of body weight to Rabbit 7 and 0.2 gm. per kilo of body weight to Rabbit 8 at 9.10 a.m. and kept them both under observation until the following day. The first few hours samples were taken every hour and later

TABLE II.

Hypoglycemia after the Injection of Guanidine Hydrochloride.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 9.10 a.m.	Blood sugar.										
				9 a.m.	10 a.m.	11 a.m.	12 n.	1 p.m.	2 p.m.	4 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.	2 p.m.
	1917	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
7	June 30	2,220	0.15	0.101	0.100	0.122	0.124	0.100	0.098	0.085	0.077	0.073	0.099	0.095
8	July 2	2,240	0.20	0.121	0.112	0.125	0.127	0.108	0.111	0.100	0.101	0.054	0.081	

at intervals of a few hours. In Rabbit 7 mild hypoglycemia was first observed 5 hours after injection and continued until the next morning, whereas No. 8 first showed a marked hypoglycemia at 8.10 p.m. which continued until the next morning when the rabbit died in convulsions.

The following group of rabbits was used to confirm the above phenomena, the blood samples being drawn at intervals of a few hours in this case. Twelve out of twenty-one showed hypoglycemia, some of them showing a reduction of more than half the normal blood sugar. Three cases were suspected but six cases show negative results. We later confirmed the fact that in these negative cases the dose was not large enough to induce hypoglycemia. The urine of the rabbits of this group was tested but none showed any sugar. In most cases hypoglycemia appears 7 hours

Digitized by Google

after the injection and usually continues several hours. Twelve of the twenty-one died following the injection; some of them several hours after injection with typical convulsions, most of them during the following night, while a few of them died within a few days. In all cases the condition of the cage showed that the animals died in convulsions. Although the cases in which hypo-

TABLE III.

First Injection of Guanidine.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar.						
				6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
23	Aug. 15	1,960	0.30	0.115		0.173				
21	" 8	1,520	0.25	0.093	0.098	0.080	0.058	0.056	0.053	
22	" 14	2,080	0.25	0.107	0.116	0.103	0.106	0.098	0.073	0.070
24	" 17	1,830	0.25	0.111		0.139	0.100	0.073	0.024	
31	Sept. 29	1,400	0.20	0.117			0.057	0.048		
30	" 29	1,600	0.20	0.115			0.080	0.072	0.068	
29	" 27	2,540	0.20	0.111			0.084	0.083	0.067	0.100
27	Aug. 24	2,600	0.20	0.120			0.053			
26	" 25	1,600	0.20	0.120			0.099	0.111	0.043	
18	" 5	1,520	0.20	0.113	0.143	0.132	0.103	0.057	0.039	0.086
14	July 22	1,740	0.20	0.120	0.123	0.104	0.069	0.074	0.057	0.105
15	" 23	1,520	0.20	0.100	0.105	0.094	0.067	0.081	0.073	0.095
19	Aug. 6	1,920	0.20	0.110	0.143	0.147	0.110	0.115	0.094	0.100
25	" 23	2,000	0.20	0.200			0.108	0.118	0.103	
28	Sept. 27	2,300	0.20	0.125			0.127	0.167	0.129	0.129
10	July 18	1,700	0.15	0.118	0.148	0.134	0.074	0.065	0.056	0.111
12	" 19	1,480	0.15	0.104	0.127	0.112	0.116	0.115	0.080	0.101
13	" 21	1,520	0.15	0.103	0.113	0.115	0.120	0.113	0.092	0.096
16	" 24	1,300	0.10	0.111	0.122	0.119	0.114	0.107	0.094	0.120
17	" 29	1,900	0.05	0.122	0.123	0.123	0.129	0.117	0.100	0.116
20	Aug. 7	1,440	0.05	0.103	0.105	0.102	0.107	0.099	0.102	0.104

glycemia occurs usually terminate fatally, three rabbits showing hypoglycemia recovered. The dose which was injected in this group varied from 0.05 gm. to 0.3 gm. per kilo of body weight. None of the rabbits that received under 0.1 gm. per kilo of body weight showed hypoglycemia and by careful inspection of the table it will be observed that the hypoglycemia provoked is almost

Digitized by Google

proportional to the dose of the drug administered. Rabbit 23, however, received the largest dose and this died before showing hypoglycemia. In the successive samples of blood from each rabbit we notice that in the first and second samples after injection of the drug the sugar content shows an increase over that in the sample taken before injection.

On the surviving rabbits a second injection of a little larger dose than the first was performed after time had been allowed for recovery from the first injection. In all cases except Nos.

TABLE IV.

Second Injection of Guanidine.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar.						
				6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	July 8	1,880	0.25	0.111	0.142	0.154	0.140	0.120	0.096	0.123
7	" 10	2,080	0.30	0.109	0.196	0.171	0.173	0.150	0.096	0.096
9	" 14	2,260	0.25	0.152		0.125	0.091	0.095	0.098	0.143
11	" 16	1,800	0.25	0.125	0.143	0.104	0.099	0.095	0.095	0.125
12	" 31	1,280	0.25	0.111	0.102	0.093	0.104	0.111	0.087	
14	Aug. 2	1,440	0.30	0.103		0.049				
16	" 3	1,320	0.20	0.136	0.176	0.170	0.171	0.136	0.101	0.116
17	" 4	1,520	0.20	0.127	0.137	0.154	0.131	0.124	0.120	0.125
18	" 12	1,220	0.30	0.109			0.074			
19	" 13	1,400	0.25	0.107		0.154	0.125	0.122	0.107	0.111
20	" 16	1,620	0.20	0.108		0.150	0.127	0.122	0.106	0.088

14 and 18 there was no hypoglycemia—the exceptions showing hypoglycemia just before they died in convulsions.

In consideration of these facts we may say that all rabbits will also show hypoglycemia after injection of the second dose of the drug, if the dose is large enough, though in this case they will die. It is interesting to note that the second injection of a large dose of guanidine provokes, in most cases, a hyperglycemia a few hours after administration and it is more marked than that appearing after the first injection. In these animals there is no glycosuria and most of them died the following night in convulsions.

A third injection was performed on the survivors of the second injection after an interval of 1 or 2 weeks. Of these only No. 11

Digitized by Google

shows hypoglycemia and this appears just before the fatal terminal convulsion. There was marked hyperglycemia for a few hours after injection in this group of rabbits and only No. 19 recovered. There was no sugar in the urine. It is worthy of note that Rabbit 19 was injected five times at intervals of 1 or 2

TABLE V.

Third Injection of Guanidine.

Rabbit No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar. 6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
3	July 12	2,500	0.25	0.122		0.169	0.145	0.143	0.109	0.119
11	" 30	2,100	0.30	0.105		0.078				
16	Aug. 10	1,120	0.30	0.108	0.200	0.204	0.148	0.131	0.122	0.111
17	" 11	1,780	0.30	0.115		0.221	0.182	0.133	0.108	0.141
19	" 28	1,880	0.25	0.121	0.200	0.167	0.137	0.124	0.111	0.104

TABLE VI.

Five Experiments with Rabbit 19.

Experiment No.	Date.	Weight.	Guanidine per kilo at 7 a.m.	Blood sugar. 6.55 a.m.	9 a.m.	11 a.m.	2 p.m.	4 p.m.	5 p.m.	8 p.m.	Next day at 9 a.m.
	1917	gm.	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	Aug. 6	1,920	0.20	0.110	0.143	0.147	0.111		0.115	0.094	0.100
2	" 13	1,400	0.25	0.107		0.154	0.125		0.122	0.107	0.111
3	" 28	1,880	0.25	0.121	0.200	0.167	0.137		0.124	0.111	0.104
4	Sept. 11	1,850	0.25	0.113	0.133	0.084	0.064		0.091	0.088	0.127
5	" 25	1,800	0.25	0.119			0.087	0.067	0.070		0.100

weeks and that no hypoglycemia occurred the first three times. The fourth and fifth injections provoked a marked hypoglycemia.

Duration of Hypoglycemia Which Is Induced by Guanidine Administration.

Usually the hypoglycemia appears 7 hours after injection of the drug and continues several hours through the night which fact makes it impossible to take samples until the next morning when

Digitized by Google

TABLE VII.

Duration of Hypoglycemia Produced by Injection of Guanidine Hydrochloride.

Rabbit 35 (2,200 gm.).				Rabbit 37 (1,820 gm.).			
Date.	Guanidine per kilo.	Blood sugar.	Remarks.	Date.	Guanidine per kilo.	Blood sugar.	Remarks.
1917	*gm.*	*per cent*		*1917*	*gm.*	*per cent*	
Oct. 16			No symptoms until 2nd	Oct. 18			
5 p.m.	0.2 at 10 p.m.	0.096	injection.	5 p.m.	0.2 at 10 p.m.	0.100	
Oct. 17				Oct. 19			
7 a.m.		0.100		7 a.m.	0.1 at 7 a.m.	0.125	Jerking backwards of head,
	0.1 at 9 a.m.						irritable, dyspnea.
10 "		0.104		9 "		0.143	
12 m.		0.111	Dyspnea, fibrillous tremor,	11 "		0.145	
			twitching of head, some-	2 p.m.		0.080	
			times convulsion.	4 "		0.070	
2 p.m.		0.091		7 "		0.054	
4 "	0.05 at 4 p.m.	0.091		10 "		0.053	
7 "		0.059		Oct. 20			
10 "		0.062		9 a.m.		0.095	Sensitive but no other
Oct. 18							symptoms.
7 a.m.		0.067	Strong convulsion.	2 p.m.		0.091	
3 p.m.		0.045	At 2.30 p.m. strong con-	Oct. 21			Very sensitive, convulsion
			vulsion, continued about	9 a.m.		0.064	continued 10 min. At 9
			10 min. Following morn-				a.m. took blood and
			ing found dead.				killed.

Digitized by Google

the sugar content has reached the normal level. Then we tried to inject at 8 p.m. and to take our first sample early in the morning. At first three rabbits were employed and after 0.1 to 0.15 gm. per kilo of body weight was injected, no change of blood sugar was observed except in No. 19 which was doubtful. The dose was probably not large enough to produce hypoglycemia. We took four more rabbits and injected 0.2 to 0.25 gm. per kilo at 10 p.m. We took our first samples the next morning but none of them showed hypoglycemia. Another 0.1 gm. per kilo was then injected into two rabbits and from 2 p.m. until the following day, over 24 hours, marked hypoglycemia was observed. Since the above dose is enough to induce hypoglycemia, the inference is drawn that the manipulation in taking the blood or the excitation of the animals accelerates the production of hypoglycemia.

The Relation of Total Solids in the Blood to the Blood Sugar Content.

The question arose whether this diminution in blood sugar was due merely to the dilution of the blood or to the effect on the sugar-regulating mechanism. To make this point clear the estimation of total solids of the blood was made.

The following table shows that when samples were taken from normal rabbits during 12 hours the variation in sugar content was much smaller than the variation in total solids.

After a few days the same rabbits were used for injecting the

TABLE VIII.

Normal Variation of Total Solid and Sugar Percentage in Blood.

Rabbit No.	Date.	9 a.m.		2 p.m.		7 p.m.	
		Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.
	1917	per cent	per cent	per cent	per cent	per cent	per cent
25	Aug. 19	0.116	16.4	0.111	16.4	0.114	15.8
26	" 20	0.115	17.4	0.105	16.0	0.108	15.1
26	" 22	0.125	15.3	0.126	14.8	0.122	13.8
27	" 21	0.114	18.8	0.108	18.3	0.110	16.6
28	Sept. 21	0.123	16.1	0.123	15.4	0.125	15.3
29	" 21	0.104	16.6	0.111	15.8	0.103	14.9
30	" 23	0.106	16.2	0.111	15.5	0.116	14.8
31	" 23	0.111	15.2	0.114	14.8	0.105	14.5
Average.		0.114	16.5	0.114	15.9	0.113	15.1

Digitized by Google

guanidine. One other rabbit was used also which later was injected twice more. One sample for comparison was taken before injection. In some animals the dose is not large enough to produce hypoglycemia. In those animals in which hypoglycemia appeared the total solids in the blood remained normal. It is worthy of note that the variation in total solids in the same rabbit is practically the same in experiments tabulated in Table VIII as in those in Table IX. In Rabbit 19 hypoglycemia appeared only after the second and third injection when the total solids remained the same as after the first injection.

TABLE IX.

Variation of Total Solid and Sugar Percentage in Blood before and after Guanidine Injection.

Rabbit No.	Date.	Guanidine injection per kilo of body weight at 7 a.m.	7 a.m.		2 p.m.		3 p.m.		4 p.m.		5 p.m.		8 p.m.	
			Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.	Sugar.	Solid.
	1917	gm.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
25	Aug. 23	0.20	0.200	15.1	0.108	16.3					0.118	15.5	0.103	14.4
27	" 24	0.20	0.120	16.9	0.053	18.5								
28	Sept. 27	0.20	0.125	15.2							0.167	14.8	0.129	14.5
29	" 27	0.20	0.111	16.5	0.084		0.087	16.0			0.083	15.4	0.067	15.0
30	" 29	0.20	0.115	16.0	0.080	15.8					0.072	14.6	0.068	13.9
31	" 29	0.20	0.117	15.5	0.057	15.9			0.048	17.5				
19	Aug. 24	0.25	0.121	15.4	0.137	15.3					0.124	14.4	0.111	13.7
19	Sept. 11	0.25	0.113	16.4	0.064	16.2					0.091	15.2	0.088	15.4
19	" 25	0.25	0.119	16.5					0.069	17.3	0.070	16.9		

Tetania parathyreopriva and idiopathic tetany are almost identical in the character of their symptoms, such as electric excitability, increased mechanical excitability, depression, emaciation, spasticity, tremor, convulsion, laryngeal spasm, etc., according to many workers. According to Burns (22) there is a striking resemblance between the metabolic evidences of tetania parathyreopriva and the injection of guanidine. Burns and Sharpe (13) demonstrated the increased guanidine base in the urine of children which manifested symptoms of idiopathic tetany and also in the urine and blood of animals which had symptoms of tetania parathyreopriva. Moreover, the serum of para-

Digitized by Google

thyroidectomized animals acts upon the muscle of frogs in the same way as do dilute solutions of guanidine (23). The administration of the salts of guanidine to animals induces symptoms almost identical with those of tetania parathyreopriva (7). The above evidence strengthens the view that a function of the parathyroids is the regulation of the metabolism of guanidine in the body and that the guanidine thus produced controls the tone of the muscle as mentioned by Paton and Findlay (24).

The results of the present investigation show that the hypoglycemia which is manifested in tetania parathyreopriva, as shown by Underhill and Blatherwick (19), can also be produced by the injection of guanidine hydrochloride. The hypoglycemia manifested after parathyroidectomy may be a secondary manifestation of guanidine poisoning.

The hyperglycemia induced several hours after administration of the drug, especially after the second and third injection, may be considered to be due to the disturbance of respiration since it is well known that dyspnea induces an increase in the sugar content of the blood.

CONCLUSIONS.

The administration of guanidine hydrochloride into rabbits induces symptoms which are almost identical with those of tetania parathyreopriva. At the same time hypoglycemia is induced which also occurs after the removal of the parathyroid. Therefore the hypoglycemia resulting from the lack of parathyroid may be due to the action of guanidine which is increased in the blood under these conditions.

The hypoglycemia induced by injection of guanidine is not due to the diminution of total solids or to the dilution of the blood.

The author acknowledges his indebtedness to Professor F. P. Underhill for suggestions and advice in carrying out this work.

BIBLIOGRAPHY.

1. Paton, D. N., and Findlay, L., *Quart. J. Exp. Physiol.*, 1916, x, 203.
2. Joseph, D. R., and Meltzer, S. J., *J. Pharm. and Exp. Therap.*, 1910–11, ii, 361.
3. Voegtlin, C., and MacCallum, W. G., *J. Pharm. and Exp. Therap.*, 1910–11, ii, 439.

Digitized by Google

4. MacCallum, W. G., and Vogel, K. M., *J. Exp. Med.*, 1913, xviii, 618.
5. Beebe, S. P., *J. Biol. Chem.*, 1907, iii, p. xxxi.
6. Beebe, *J. Biol. Chem.*, 1909, vi, p. xiv.
7. Paton and Findlay, *Quart. J. Exp. Physiol.*, 1916, x, 315.
8. Kutscher and Lohmann, *Z. physiol. Chem.*, 1906, xlix, 81.
9. Engeland, R., *Z. physiol. Chem.*, 1908, lvii, 49.
10. Achelis, W., *Z. physiol. Chem.*, 1906–07, l, 10.
11. Koch, W. F., *J. Biol. Chem.*, 1912, xii, 313.
12. Koch, *J. Biol. Chem.*, 1913, xv, 43.
13. Burns, D., and Sharpe, J. S., *Quart. J. Exp. Physiol.*, 1916, x, 345.
14. Hirsch, R., *Z. exp. Path. u. Therap.*, 1906, iii, 393; 1908–09, v, 233.
15. Eppinger, H., Falta, W., and Rudinger, C., *Wien. klin. Woch.*, 1908, xxi, 241; *Z. klin. Med.*, 1908, lxvi, 1; 1909, lxvii, 380.
16. Pari, G. A., *Biochem. Z.*, 1908, xiii, 281.
17. Underhill, F. P., and Saiki, T., *J. Biol. Chem.*, 1908–09, v, 225.
18. Underhill, F. P., and Hilditch, W. W., *Am. J. Physiol.*, 1909–10, xxv, 66.
19. Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.
20. Underhill and Blatherwick, *J. Biol. Chem.*, 1914, xix, 119.
21. McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.
22. Burns, D., *Quart. J. Exp. Physiol.*, 1916, x, 361.
23. Wishart, G. M., *Quart. J. Exp. Physiol.*, 1916, x, 355.
24. Paton and Findlay, *Quart. J. Exp. Physiol.*, 1916, x, 377.

Digitized by Google

Digitized by Google

MATHEMATICAL FORMULAS AND ACID EXCRETION.

By GEORGE D. BARNETT.

(From the Medical Division of the Stanford University Medical School, San Francisco.)

(Received for publication, December 5, 1917.)

The application of mathematical formulas in the attempt to elucidate biological processes has lately furnished us with laws, quantitative relationships, constants, and indices which might seem to leave little to be desired. Some of us, however, have been inclined to question the value of certain of these laws, particularly as applied to the excretion of a variety of substances by the kidney, not only on account of the positive contrary evidence brought forward, but also on the ground that we believe it improbable that our present knowledge is sufficient to permit us to express the complexities of kidney activity with mathematical accuracy.

An analysis of the data presented in the recent paper of Fitz and Van Slyke[1] on acid excretion affords a specific basis for criticism of one such mathematical relationship. In this paper the authors search for a "quantitative relationship . . . . between the alkali reserve of the blood plasma, as measured by the combining power for CO_2, and the rate of acid excretion by the kidneys," and arrive at the conclusion that the combined formula developed by Ambard for urea and for chloride excretion is applicable here in the form

$$\text{Plasma } CO_2 = 80 - \sqrt{\frac{D}{W}\sqrt{C}}$$

where D is the excretion rate for acid plus ammonia, C their concentration in the urine, and W the weight of the individual. They adopt this form as an expression of the true quantitative relationship only after trials of various modifications, concluding

[1] Fitz, R., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 389.

Digitized by Google

that it holds "more consistently than any other expression which could be found in the literature or devised." In their development they note, for instance, that the fourth root of C has been retained because the square root gave too much and the sixth root too little influence to the factor C. Now since it does not appear from any ordinary inspection of the values of C given that it can have any appreciable influence, it would seem logical to revise the formula, replacing C by a constant. Thus it is possible to arrive at the formula

$$\text{Plasma } CO_2 = 80 - 5\sqrt{\frac{D}{W}}$$

whose calculated values for plasma CO_2 for the 76 observations given show an average deviation from the found values of 5.45 volumes per cent compared with 6.99 volumes per cent by the original formula. That is to say, the results average 1.5 volumes per cent better if instead of using the fourth root of C we use the figure 5. Therefore we must conclude that the "influence" of C in this formula is negligible, and that consequently the corollary statement made by the authors that "other factors being the same, the amount of acid excreted in excess of mineral bases is increased, on the average, as the square root of the volume of urine" must also be revised; for it is apparent that as far as we may conclude from this equation the acid excretion is quite independent of the volume of urine.

We may further omit W, and devise the form

$$\text{Plasma } CO_2 = 80 - 0.7\sqrt{D}$$

which will also give results more closely approximating the found values of CO_2 than those derived from the Ambard type of formula.

Or if we wish to make use of the ammonia excretion rate only, it will be found that from the equation

$$\text{Plasma } CO_2 = 80 - 0.9\sqrt{NH_3}$$

(where NH_3 is the amount of 0.1 N ammonia excreted per 24 hours) we can likewise calculate the index of blood bicarbonate with considerably greater precision and with much less mathe-

Digitized by Google

matical effort. And so on. It would no doubt be possible to evolve many other forms equally applicable. The following table summarizes the comparative merits of the Ambard type of formula and the three modifications suggested.

Formula.	Average deviation. *vol. per cent*	No. of errors greater than 10 volumes per cent.
$CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$	6.99	15
$CO_2 = 80 - 5\sqrt{\frac{D}{W}}$	5.45	11
$CO_2 = 80 - 0.7\sqrt{D}$	5.85	12
$CO_2 = 80 - 0.9\sqrt{NH_3}$	6.35	11

While it is thus apparent that many formulas can be derived which show a certain degree of applicability to the facts of acid excretion, it does not at all follow that they are to be regarded as true quantitative relationships. Certainly before we accept any formula as such we must justify it further than by the mere statement that "the margin of error to be accepted in this instance appears to be about 10 volumes per cent of plasma CO_2." For certainly if the relationship be a true one this margin of error must bear some fairly close relation to the deviation produced by possible errors in the original observations. Suppose, then, in the original formula above, we allow an error of 5 per cent in determining urinary acidity, a similar error in determining ammonia, 2.5 per cent error each in measuring urinary volume and the weight of the individual, and 6 per cent error in determining plasma bicarbonate, all of which are much greater than the errors of the methods involved. If such maximal errors occurred in *every* urinary, weight, volume, and CO_2 determination in the entire series and always occurred in such a manner as to make their effects additive, the error deviation of the calculated from

Digitized by Google

the found CO_2 would be less than 5 volumes per cent,[2] or considerably less than the *average* deviation found by the best of the above laws.

From this brief analysis we must therefore conclude that the Ambard type of formula cannot be regarded as a quantitative relationship between the variables of acid excretion, and that no mathematical expression so far devised can lay claim to even a moderate degree of accuracy as such. Furthermore, it seems obvious that such formulas constantly give the impression of mathematical accuracy where none exists, and that their use is apt to prove misleading to the many who are inclined to accept them without question.

SUMMARY.

In criticism of the application of the Ambard type of formula to acid excretion, it is shown that:

1. Substitution of constants for certain of the variables leads to an improvement in the calculated results.

2. The average deviation of calculated from found values of plasma CO_2 is much greater than would result from maximal additive errors in all the determinations involved.

[2] Under the influence of the assumed errors the quantity $\sqrt{\frac{D}{W}\sqrt{C}}$ becomes $\sqrt{\frac{1.05\,D}{0.975\,W}\sqrt{\frac{1.05\,D}{0.975\,W}}}$, simplifying which we obtain

$$\left(\frac{1.05}{0.975}\right)^{\frac{1}{2}}\sqrt{\frac{D}{W}\sqrt{C}},\ \text{or}\ 1.06\sqrt{\frac{D}{W}\sqrt{C}}.$$

From this source the error in volumes per cent of calculated CO_2 will thus be 6 per cent of $\sqrt{\frac{D}{W}\sqrt{C}}$. Allowing also a 6 per cent error in the CO_2 determination, the greatest possible maximal deviation will be $0.06\left(CO_2+\sqrt{\frac{D}{W}\sqrt{C}}\right)$, and since by the given law $CO_2+\sqrt{\frac{D}{W}\sqrt{C}}=80$, this becomes 4.8 volumes per cent.

Digitized by Google

STUDIES OF ACIDOSIS. X.

By DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, December 31, 1917.)

The present paper includes a reply to Barnett's (1) critique in the preceding article and a summary showing the nature of the results obtained with the methods for estimating alkaline reserve from urine and alveolar air analyses.

Fitz and the writer (2) succeeded recently in demonstrating a quantitative relationship between the alkaline reserve and the excretion of acids in excess of fixed base, as measured by ammonia plus titratable acid in the urine. The empirical formula utilized to demonstrate the relationship was, plasma $CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$, D representing the cc. of 0.1 N titratable acid and ammonia excreted per 24 hour time unit, and C (concentration) the amount excreted per liter.

Barnett states the belief that this formula does not express the relationship between plasma bicarbonate and acid excretion with sufficient accuracy to justify its use. The reason for this opinion is that the results show that the average error in calculating the plasma bicarbonate from the excretion exceeds that which would result from maximal analytical errors in all of the determinations involved. This seems to us insufficient ground for the criticism. The essential question, for our purpose, is not whether the plasma bicarbonate can be estimated from urine excretion with no error except that of the chemical determinations, but whether the total sum of the errors of analysis, individual variation in kidney function, and fault in the empirical formula is within such limits that the excretion may be of any assistance in estimating the alkaline reserve when conditions prevent the direct determination of the latter in the blood.

We have given in Papers IV (2) and VI (3) data which show the limits of accuracy encountered in estimating plasma bicar-

Digitized by Google

bonate from acid excretion in practically every form of diabetic acidosis. It does not appear to us that a valid reason has been advanced for modifying the conclusion based on those data; *viz.*, that the ammonia plus acid excretion is quantitatively related to the bicarbonate deficit in the blood, and that the relationship is sufficiently uniform to be useful in estimating this deficit when the limitations of accuracy, as shown by our data, are taken into consideration.

Barnett's claim that all the variables of the formula except the two most important ones, the blood bicarbonate and ammonia excretion, may be replaced by a constant without significantly diminishing the average accuracy of the formula, would likewise appear to us, even if entirely justified, to detract nothing from the correctness of the above conclusion.

Any simplification in the calculation not resulting in loss of accuracy would be an improvement, however, and therefore it is desirable to examine somewhat closely the formula minus one, two, and three of its variables in order to decide how many of them may be deleted without lessening the reliability of results.

Concerning the dropping of C, thereby simplifying the formula

$$\text{Plasma } CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C} \text{ to, Plasma } CO_2 = 80 - 5\sqrt{\frac{D}{W}},$$

we agree with Barnett that it makes the equation not only simpler, but also measurably more accurate and is therefore in every way desirable.[1] In preliminary experiments in which we tested several formulas before one was chosen to apply systematically, the value of C appeared to have an appreciable effect, although much less than that of D; *i.e.*, other conditions being the same, a greater volume of urine appeared to carry out somewhat

[1] From the negligible average influence of C it appears that, at least as long as either normal or exceptionally high volumes of urine are excreted (which was the case with all our patients), variations in the volume are of no effect on the acid excretion. We are not, however, prepared to follow this conclusion as to the non-effect of C to its logical limit and state that in a patient with severe diabetic acidosis flushing with water is of no value. A liter of urine is not an abnormally small amount in a normal adult, but it could carry out only about 15 gm. of β-hydroxybutyric acid, if, as indicated by the data of Magnus-Levy and others, this acid is never excreted in concentration greater than 1.5 per cent. To remove the

Digitized by Google

more acid than a smaller volume. We accordingly indicated this effect by introducing C into the formula, and indicated its comparatively minor importance by using its fourth root. The formula thus involved enabled us to solve our main problem; *viz.*, the question as to whether any quantitative relationship could be demonstrated between fall in alkaline reserve and rise in ammonia and acid excretion. The formulas which had been discarded in the preliminary tests we did not afterwards apply to the main body of our data. It is fortunate that Barnett has now retested the formula without C on our published data, and shown that this variable may be neglected. With the formula thus simplified it becomes possible, as indicated in the accompanying table, to interpret the 24 hour excretion of acid plus ammonia directly into terms of acidosis with the use of no more elaborate terms than $\frac{D}{W}$, or cc. of 0.1 N acid plus ammonia per kilo.

Barnett also calculates from the results of Paper IV that but little is gained in average accuracy by including variations in the body weight in the estimation. The data of Paper IV, however, taken without those of Paper VI, are not suited to decide statistically the question of the influence of body size. Of the 65 determinations on diabetics reported in Paper IV, 29 are on a single patient of 50 kilos weight, and of the others, only one determination was made on a subject of less than 37 or more than 50 kilos weight who had a marked acidosis without bicarbonate dosage. In this one, a boy of 12 in actual coma, both the original $\sqrt{\frac{D}{W}}\sqrt{C}$ and the 5 $\sqrt{\frac{D}{W}}$ formula indicate a plasma CO_2 of 30 per cent, which is a severe acidosis, though not so severe as that shown both by clinical con-

much larger amounts sometimes formed, excretion of several liters of urine per 24 hours appears necessary. The formation of large amounts of acetone bodies, or of the sugar which accompanies them, apparently acts as a diuretic, and secures the necessary excretion. For example, Magnus-Levy reports that when a patient on 2 successive days excreted 109.5 and 157 gm. of organic acid calculated as hydroxybutyric, the corresponding urine volumes were 8.0 and 9.2 liters (Magnus-Levy (4), p. 182). Similar high excretions are seen in the data of Papers IV and VI when acid excretion, as indicated by the ammonia plus titratable acid, was high.

Digitized by Google

dition and plasma CO_2 of 14 per cent. If the body weight were neglected in the calculation, however, and Barnett's $0.7\sqrt{D}$ formula used, the excretion would indicate a plasma CO_2 of 46, or almost no acidosis. Similar magnification of error is introduced in attempting to interpret the excretion data regardless of body weight in the two patients of less than adult size with acidosis reported in Paper VI. These are No. 3, a boy of 12 with intense acidosis, and No. 5, a boy of 13 with severe acidosis. That an allowance for body size must be made in interpreting the rate of formation or excretion of any metabolic product is a generalization so well founded that previous discussion of it seemed unnecessary.

Elimination of a third variable, the titratable acid, from the calculation, would apparently be a further step backwards. It is true, as exemplified by our own data, that ammonia and titratable acid in diabetic urine as a rule rise and fall together, the ammonia being usually two to three times the titratable acid. The ratio $\frac{0.1\text{ N }NH_3}{0.1\text{ N acid}}$ is by no means constant, however, varying from 0.3 to 5.0 in diabetic and normal urines, so that the titratable acid sometimes exceeds the ammonia. Since both ammonia and titratable acid indicate excretion of acid in excess of fixed base, it is does not seem logical to determine the one and neglect the other. The result of neglecting the titratable acid is apparent, except in the case of the one diabetic who was chosen for continuous observation, in a decided increase in the average error.

Average Errors. Data of Paper IV.

Formula.	Table I. 11 normal persons.	Table II. 36 different diabetics.	Table III. 29 observations on 1 diabetic.
$CO_2 = 80 - 5\sqrt{\frac{D}{W}}$	2.5	5.4	5.7
$CO_2 = 80 - 0.9\sqrt{NH_3}$	4.6	7.4	5.7

A practical additional reason for determining titratable acid as well as ammonia is that it protects against a false diagnosis of acidosis which might be made from the ammonia alone in urines

Digitized by Google

that have undergone bacterial decomposition, either in the bladder as the result of cystitis, or outside the bladder as the result of preservation with insufficient antisepsis. As long as the ammonia formation leaves the urine still acid, it does not much alter the NH_3 + acid figure, since CO_2, of the ammonium carbonate formed by bacterial action on urea, escapes, while the ammonia remains and neutralizes approximately an equivalent of acid. The net effect of an increase of ammonia is therefore an approximately equal decrease of titratable acid, with no significant influence on the resultant sum of the two.

Our data indicate furthermore that the ratio $\frac{0.1\ \text{N}\ NH_3}{0.1\ \text{N acid}}$ may be used as a fairly sensitive indicator of ammoniacal decomposition. In the urines of Paper IV, which were all analyzed while perfectly fresh, the ratio varies from 0.3 to 4.8 averaging 1.6 in normal men and 2.3 in diabetics. In only one case was a value of 4.1 exceeded. It appears therefore that when the ammonia exceeds four or five times the titratable acid there is ground for suspecting the origin of a measurable portion of the ammonia in bacterial action. Although the 24 hour urines reported in Paper VI are believed to have been collected with at least ordinary care and were preserved with toluene, it will be seen from the results that in these urines the ammonia:acid ratio averages higher (about 4.1) than in the quickly collected and analyzed specimens of Paper IV, and frequently exceeds the maximum of the short-time urines, at times rising as high as 8 or 9. In no case had decomposition gone so far as to neutralize all or nearly all of the titratable acid, so that the results, based on the sum of ammonia and titratable acid, could not have been significantly affected. The frequency of high ammonia:acid ratios in the 24 hour urines nevertheless indicates the readiness with which decomposition may occur in 24 hour specimens even when collected with routine precaution.

For the above reasons we believe that when diabetic acidosis must be estimated from acid excretion, the most satisfactory formula at present available for expressing the results in terms of alkaline reserve is

$$\text{Plasma } CO_2 = 80 - 5\sqrt{\frac{0.1\ \text{N (acid} + NH_3\text{) per 24 hours}}{\text{kilos body weight}}}$$

Digitized by Google

and that neither the titratable acid nor body weight may be neglected without increasing the chance of error in the estimation.

As, thanks to Barnett, the acid excretion formula is simplified to the above by elimination of one unnecessary variable, and as Palmer and Van Slyke in Paper IX (5) have published data which add the bicarbonate retention to the indirect acidosis tests that have been composed with the direct, it appears desirable to revise accordingly the summarizing table on page 412 of Paper VI. In the following table we have therefore substituted the simpler expression for acid excretion and have added the data for the bicarbonate retention test. We have also indicated the chief fallacies to which, according to the results published in the present series of papers, each indirect test is liable when applied to the detection of diabetic acidosis.

It should be noted that the data obtained from kidney and lung excretion as measures of alkaline reserve in diabetic acidosis do not necessarily hold for other types of acidosis. In nephritis, for example, the two tests based on kidney excretion become fallacious, while Peters (6) has recently shown that in cardiac dyspnea and in conditions involving great diminution of lung capacity the mechanics of respiration are so disturbed that the alveolar carbon dioxide ceases to be an approximate measure of blood bicarbonate. The indirect tests may be trusted as approximate indicators of alkaline reserve only in conditions where they have been previously tested by comparison with the blood bicarbonate. We have made this comparison in diabetes, but the results do not hold for other pathological conditions.

SUMMARY.

Acid excretion as a measure of diabetic acidosis is, according to present data, most significantly expressed in terms of ammonia plus titratable acid per unit of body weight. The average error involved in estimating alkaline reserve from acid excretion is, as shown by Barnett, appreciably reduced by simplifying our original empirical formula, plasma $CO_2 = 80 - \sqrt{\frac{D}{W}}\sqrt{C}$, to plasma $CO_2 = 80 - 5\sqrt{\frac{D}{W}}$. The absolute difference in the re-

Digitized by Google

Condition of subject.	Actual bicarbonate reserve. Plasma bicarbonate CO_2 reduced to 0°, 760 mm.	Corresponding results of indirect tests for acidosis.					
		24 hour excretion* of 0.1N acid + NH_3.		Carbon dioxide of alveolar air.		Sodium bicarbonate required to turn urine alkaline.	
		(a) Cc. per kg. (b) Approximate cc. per 60 kg. person.	Reliability in diabetes.	(a) Mm. tension. (b) Approximate per cent.	Reliability in diabetes.	(a) Gm. per kg.‡ (b) Approximate gm. for a 60 kg. person.	Reliability in diabetes.
	vol. per cent	*cc.*					
Normal resting adult. Extreme limits of bicarbonate reserve.	80–53	(a) 0–27 (b) 0–1,600	Good.	(a) 53–35 mm. (b) 6.8–4.7 per cent.	May indicate some acidosis in its absence.	(a) 0–0.5 (b) 0–30	May indicate acidosis in its absence.
Mild acidosis, no pronounced symptoms.	53–40	(a) 27–65 (b) 1,600–4,000	Good.†	(a) 35–27 mm. (b) 4.7–3.6 per cent.	May indicate more acidosis than is present.	(a) 0.5–0.8 (b) 30–50	May indicate much more acidosis than is present.
Moderate to severe acidosis. Symptoms may be apparent.	40–30	(a) 65–100 (b) 4,000–6,000	Liable† to considerable error in either direction.	(a) 27–20 mm. (b) 3.6–2.7 per cent.	Good.	(a) 0.8–1.1 (b) 50–65	" "
Severe acidosis. Symptoms of acid intoxication.	Below 30	(a) Over 100 (b) " 6,000	" "	(a) Below 20 mm. (b) 2.7 per cent.	"	(a) Over 1.1 (b) Over 65	" "

* Measured either in 24 hour urine or on specimen from shorter period calculated to 24 hour basis.

† After bicarbonate administration likely to indicate more acidosis than is present.

‡ The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkaline reserve to normal from acidosis of the severity indicated by the corresponding plasma CO_2 figures in the first column.

Digitized by Google

sults calculated by the two equations is, however, so small that the remarks on the range of error in such calculations made in Papers IV and VI hold with essentially equal force when the simplified formula is used. Further simplification, by neglecting the body weight or titratable acid as suggested by Barnett, decreases the accuracy of the estimation.

For practical purposes the acid excretion may, without going through the calculation of the formula, be interpreted directly into terms of clinical severity of acidosis, as indicated in the table; *e.g.*, an excretion exceeding 27 cc. of 0.1 N ammonia plus acid per kilo indicates acidosis, which usually becomes critical if the excretion approaches 100 cc. per kilo.

The relationships of the plasma bicarbonate to acid excretion, alkali retention, and alveolar carbon dioxide tension are summarized for reference in a table, wherein are also indicated the chief errors to which, according to the data of Papers IV, VI, and IX of this series, the three latter determinations are subject as measures of diabetic acidosis.

BIBLIOGRAPHY.

1. Barnett, G. D., *J. Biol. Chem.*, 1918, xxxiii, 267.
2. Fitz, R., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 389. Fitz' collaboration in the present paper is prevented by his absence on military service.
3. Stillman, E., Van Slyke, D. D., Cullen, G. E., and Fitz, R., *J. Biol. Chem.*, 1917, xxx, 405.
4. Magnus-Levy, A., *Arch. exp. Path. u. Pharm.*, 1899, xlii, 149.
5. Palmer, W. W., and Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxxii, 499.
6. Peters, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 118.

Digitized by Google

THE EFFECTS OF ELECTROLYTES ON GELATIN AND THEIR BIOLOGICAL SIGNIFICANCE.

I. THE EFFECTS OF ACIDS AND SALTS ON THE PRECIPITATION OF GELATIN BY ALCOHOL.

By W. O. FENN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, December 21, 1917.)

The biologist can hardly hope to find a complete explanation of the complicated effects of electrolytes upon protoplasm until the chemist can give him an explanation of their almost equally complicated effects upon the purified constituents of protoplasm. In order to obtain more complete information concerning the effects of electrolytes upon proteins, the precipitation of gelatin by alcohol has been studied. This property of gelatin is more simple to measure and more sensitive to small variations in the salt content of the solution than its viscosity, swelling, or temperature of gelation. This method has yielded, therefore, more complete data than have hitherto been obtained. Pending a more detailed inquiry into the reactions involved, it is deemed advisable to publish the facts obtained, omitting the theoretical interpretation.

Description of the Method.

The method which was worked out for the investigation has already been briefly described[1] together with some of the results which are of particular interest to the biologists. It consists essentially in adding 95 per cent alcohol to 5 cc. of the gelatin-electrolyte mixture until an opaque precipitate is produced. The sharpness of the end-point varies characteristically with the particular electrolyte and the particular concentration used but can ordinarily be determined to within 0.1 cc., which is

[1] Fenn, W. O., *Proc. Nat. Acad. Sc.*, 1916, ii, 534, 539.

Digitized by Google

accurate enough for the purpose. Two determinations were made for each point and the average was taken as the true value. The test-tubes containing the samples to be titrated with alcohol were kept at constant temperature for some hours before using in order to reach equilibrium. The time allowed for this and the temperature chosen varied considerably in the different experiments but, in plotting, only curves which are comparable in all essential respects are placed together, unless mention is made to the contrary. In mixing, the gelatin was dissolved in water at 90°C. and 5 cc. of the resulting solution were mixed with 5 cc. of the solution of the electrolyte to be investigated. The two halves, of 5 cc. each, of these mixtures were used for the two determinations of each point. Three grades of commercial gelatin were used throughout. Gelatin A, which was used in all except the earlier experiments, set at a lower concentration and gave a clearer solution than gelatin B or C. The concentration of the gelatin was so chosen (2 or 3 per cent) that it would not set at the temperature of the experiment. Throughout this paper, the number of cc. of 95 per cent alcohol necessary to produce an opaque precipitate in 5 cc. of the gelatin-electrolyte mixture will be spoken of as the "alcohol number" for the sake of brevity. In interpreting the results, it should be remembered that in titrating, both the gelatin and the electrolyte are being diluted, and the solution is being cooled by the addition of the alcohol. The alcohol number is ordinarily increased by a rise in temperature and by dilution of the gelatin. The cooling effect of the alcohol could be avoided by allowing the gelatin to reach equilibrium at room temperature, but sharper end-points are obtained by using stronger gelatin. This necessitates a higher temperature to prevent setting. If, in titrating, the alcohol is run in too rapidly, the value obtained for the alcohol number will be too high as the precipitate takes a certain short time to form. This error is important only in the case of NaOH and HCl and similar electrolytes. The gelatin is stirred after each addition of alcohol. The end-point is defined as the point where a pencil held behind the test-tube can no longer be seen.

The alcohol number is ordinarily increased by a rise in temperature but in the presence of Na_2SO_4 in concentrations greater than 0.2 M and NaCl M or stronger, the reverse is true.

Digitized by Google

The results obtained by this method may be presented under the following four headings: (I) The effect of single electrolytes. (*a*) Acids and alkalies; (*b*) salts. (II) The effect of combinations of salts with acids and alkalies. (III) The effect of combinations of salts. (IV) The precipitation of gelatin by mixtures of electrolytes without alcohol. Each of these will be presented in a separate paper.

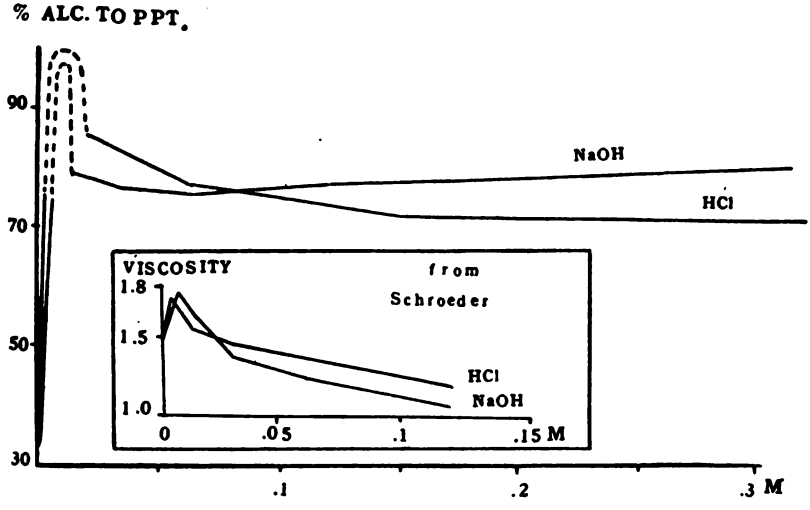


FIG. 1. Curves showing the effect of NaOH and HCl on the precipitation of gelatin by alcohol. The ordinates represent the concentrations of alcohol in the solution at the point of precipitation, and the abscissæ the concentrations of HCl and NaOH at that point. The dotted lines indicate that no precipitate could be obtained no matter how much alcohol was added. The insert shows von Schroeder's curves for the viscosity of gelatin sols, showing that the alcohol maximum corresponds to a viscosity maximum.

a. Acids and Alkalies.

The presence of acids and alkalies in gelatin hinders the precipitation by alcohol. With strong acids and alkalies, like NaOH and HCl, this effect passes through a maximum.

The effects of NaOH and HCl are shown in Fig. 1, where the concentration of alcohol at the point of precipitation is plotted (ordinates) against the concentration of NaOH or HCl at the end-

Digitized by Google

point (abscissæ). The maximum in the curves corresponds to a point of maximum swelling and maximum viscosity. Von

TABLE I.

Effect of HCl and NaOH on the Precipitation of Gelatin by Alcohol.

HCl				NaOH			
Concentration of HCl at start.	Alcohol to precipitate.	Concentration of HCl at end-point.	Alcohol (by volume) at end-point.	Concentration of NaOH at start.	Alcohol to precipitate.	Concentration of NaOH at end-point.	Alcohol (by volume) at end-point.
M	cc.	M	*per cent*	M	cc.	M	*per cent*
12.2	55	1.0	85	4.9	No ppt.		
6.1	30	0.85	79	3.26	" "		
3.05	19	0.61	72	2.17	33	0.38	81
1.52	18	0.32	71	1.45	29	0.26	79
0.76	19	0.15	72	0.96	28	0.17	78
0.38	25	0.063	77	0.64	26	0.12	77
0.19	50	0.018	85	0.32	23	0.064	75
0.095	No ppt.			0.16	24	0.032	76
0.048	" "			0.08	30	0.014	79
0.024	" "			0.04	No ppt.		
0.012	23	0.0021	75	0.02	" "		
0.006	9	0.002	57	0.01	16	0.0027	69
0.003	6	0.0013	47	0.005	8	0.0021	54
0.0015	4	0.00075	37	0.0025	6	0.0012	47.5
0.0	3		32	0.00125	5	0.0007	43
				0.000625	4.5	0.00035	41
				0.0	3.2	0.0	33

5 cc. of the HCl and NaOH solutions, in the concentrations noted in the first columns, were placed in each tube. 1 cc. of 7 per cent gelatin B was then added to each. HCl and NaOH are so effective in preventing precipitation that the titration with alcohol must be carried on slowly in order to allow the precipitate time to form. In order to save time, all the tubes were titrated simultaneously. The same number of cc. of alcohol was added to each in turn until precipitation occurred. The experiments were performed at room temperature. The decrease of precipitability in high concentrations is due to decomposition of the gelatin. For purposes of plotting, the concentrations of HCl, NaOH, and alcohol at the point of precipitation are calculated. These data are plotted in Fig. 1.

Schroeder's curves for the viscosity of gelatin sols are plotted on the insert for comparison.[2] Their essential similarity to the alcohol curves is undoubted.

[2] von Schroeder, P., *Z. physikal. Chem.*, 1903, xlv, 75.

Digitized by Google

Similar curves have been worked out for various other acids. Lactic, trichloroacetic, acetic, and formic acids give curves which rise more gradually than the HCl curve. It is impossible to follow them through the maximum as no precipitate can be obtained. Phosphoric acid gives a sharp fall after the maximum which can be followed, but no precipitate can be obtained at the maximum. Tartaric acid and sulfuric acid rise slowly to a comparatively low maximum and do not drop thereafter. In general, the weak acids have less effect and show either no maximum or less maximum than the HCl curve. The small effect of H_2SO_4, compared to HCl and HNO_3, is probably due to the effect of the bivalent SO_4 anion.

The fact that acids and alkalies prevent the precipitation of proteins by alcohol was shown by Pauli and Handovsky[3] and by Schorr,[4] although in neither case was the complete course of the curve worked out. Pauli and collaborators,[5] Lacqueur and Sackur,[6] and Zoja[7] have shown that acids and alkalies increase the viscosity of protein sols, and von Schroeder[2] has shown the same for gelatin sols. The swelling of gelatin has been measured by Ostwald,[8] Procter,[9] Fischer,[10] and Ehrenberg.[11] The characteristic maxima in both acids and alkalies, as found by these workers, correspond to the maxima of the alcohol curves as found by the writer. Ostwald, however, finds in addition an initial minimum (in swelling) which is more pronounced in acids but discernible in alkalies also. In spite of the overwhelming evidence to the contrary of Pauli and his pupils, he attempts to homologize this minimum with

[3] Pauli, W., and Handovsky, H., *Biochem. Z.*, 1909, xviii, 340.

[4] Schorr, C., *Biochem. Z.*, 1911, xxxvii, 424.

[5] Pauli, W., *Z. Chem. u. Ind. Kolloide*, 1908, iii, 2; 1910, vii, 241; 1913, xii, 222; *Tr. Faraday Soc.*, 1913, ix, 54. Pauli, W., and Wagner, R., *Biochem. Z.*, 1910, xxvii, 296. Pauli, W., and Falek, O., *ibid.*, 1912, xlvii, 269.

[6] Lacqueur, E., and Sackur, O., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 193.

[7] Zoja, L., *Z. Chem. u. Ind. Kolloide*, 1908, iii, 249.

[8] Ostwald, W., *Arch. ges. Physiol.*, 1905, cviii, 563; cix, 277; 1906, cxi, 581; *Tr. Faraday Soc.*, 1913, ix, 34.

[9] Procter, H. R., *Kolloidchem. Beihefte*, 1910–11, ii, 243. Procter, H. R., and Wilson, J. A., *J. Am. Leather Chem. Assn.*, 1916, xi, 261. Procter, *J. Chem. Soc.*, 1914, cv, 313. Procter and Wilson, *ibid.*, 1916, cix, 307.

[10] Fischer, M. H., Œdema and Nephritis, New York, 2nd edition, 1915.

[11] Ehrenberg, R., *Biochem. Z.*, 1913. liii, 356.

Digitized by Google

von Schroeder's maximum viscosity. It is difficult to understand this unless Ostwald has confused the "hydration viscosity" as measured by von Schroeder in dilute solutions with the "gelation viscosity" of solutions which are strong enough to set. The gelation viscosity is of course at a maximum in an approximately neutral solution. It is evident that Ostwald's minimum must be some secondary phenomenon inherent in the swelling method.

The equilibrium between proteins and acids has been studied in more detail by other methods. Bugarsky and Liebermann[12] and Manabe and Matula[13] have made simultaneous electrometric determinations of the H and Cl ions combined with the protein in HCl. The amount of HCl combined with the protein has been determined by Van Slyke[14] by conductivity measurements, and the amount of the H ion combined has been determined by Pauli and Hirschfeld[15] and by Procter[9] by potentiometer measurements of the H ion concentration. The latter has made perhaps the most notable contribution to the subject by giving a quantitative explanation of the maximum swelling of gelatin in acids.

b. Salts.

The following conclusions can be drawn concerning the effects of salts on the alcohol number of gelatin. (1) All salts increase the alcohol number of gelatin except certain ones which combine a bivalent or trivalent cation with a bivalent or trivalent anion. (2) Trivalent anions and cations are more effective than bivalent, and bivalent are more effective than monovalent in hindering precipitation. (3) The lyotropic effect is of minor importance except in high concentrations of sulfates, citrates, and tartrates.

The results of the experiments with salts alone are plotted in Figs. 2 to 5 and the data are given in the corresponding tables. In all cases the ordinates represent the number of cc. of alcohol required to produce a precipitate in 5 cc. of the gelatin-salt mix-

[12] Bugarsky, S., and Liebermann, L., *Arch. ges. Physiol.*, 1898, lxxii, 51.

[13] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

[14] Van Slyke, L. L., and Van Slyke, D. D., *Am. Chem. J.*, 1907, xxxviii, 383.

[15] Pauli, W., and Hirschfeld, M., *Biochem. Z.*, 1914, lxii, 245.

Digitized by Google

TABLE II.

Effect of Various Chlorides (and Other Salts) on the Precipitation of Gelatin by Alcohol.

NaCl		$CuCl_2$		$AlCl_3$		$CaCl_2$		$CuSO_4$		$MgCl_2$		$MnSO_4$	
Concentration of NaCl.	Alcohol to precipitate.	Concentration of $CuCl_2$.	Alcohol to precipitate.	Concentration of $AlCl_3$.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.	Concentration of $CuSO_4$.	Alcohol to precipitate.	Concentration of $MgCl_2$.	Alcohol to precipitate.	Concentration of $MnSO_4$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
2.0	13.5	1.0	13.4	0.0022	No ppt.	0.52	14.45	0.665	3.5	0.48	13.55	0.5	3.6
1.0	12.2	0.5	13.5	0.0011	6.85	0.26	14.45	0.333	3.6	0.24	12.0	0.25	4.1
0.5	10.85	0.25	14.8	0.00556	5.55	0.13	14.8	0.167	3.35	0.12	10.5	0.125	4.15
0.25	9.45	0.125	19.35	0.000278	4.95	0.065	14.55	0.083	3.05	0.06	10.2	0.0625	4.25
0.125	8.4	0.0625	No ppt.	0.000139	4.65	0.0327	15.1	0.042	3.25	0.03	9.0	0.0312	4.3
0.0625	7.47	0.0312	" "	0.0	4.4	0.0164	12.65	0.021	3.6	0.015	8.05	0.0156	4.1
0.0312	6.6	0.0156	" "			0.0082	10.1	0.0105	4.05	0.0075	7.0	0.0078	4.4
0.0156	6.05	0.0078	" "	Temperature about 30°C.		0.0041	7.8	0.005	4.45	0.00375	6.2	0.0039	4.2
0.0078	5.85	0.0039	" "			0.002	6.5	0.0025	4.7	0.00138	5.4	0.0	4.3
0.0039	5.45	0.00195	7.35			0.001	5.75	0.0013	4.6	0.00069	4.9	Temperature 30°C.	
0.00195	5.3	0.0	4.75			0.0	5.1	0.0	4.6	0.00034	4.75		
0.0	5.1					Temperature 30°C.		Temperature 31°C.		0.0	4.5		
Temperature 30°C.		Temperature about 31°C.								Temperature 31°C.			

For each salt the first column shows the molecular concentration present in the gelatin before the addition of alcohol. The second column shows the number of cc. of 95 per cent alcohol which was added to 5 cc. of the gelatin-salt mixture in order to produce an opaque precipitate; *i.e.*, the alcohol number. Two determinations were made for each point and the average is given in the tables. The concentration of the gelatin before titration was 2 per cent.

Digitized by Google

ture, and the abscissæ the concentration of salt in the gelatin before titration with alcohol.

In general, all the curves show that with increasing concentra-

TABLE III.

Effect of Various Salts on the Precipitation of Gelatin by Alcohol.

$MgCl_2$.		$CaCl_2$		$MnCl_2$		$Mg(NO_3)_2$		$SrCl_2$	
Concentration of $MgCl_2$.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.	Concentration of $MnCl_2$.	Alcohol to precipitate.	Concentration of $Mg(NO_3)_2$.	Alcohol to precipitate.	Concentration of $SrCl_2$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
0.5	11.1	2.85	11.85	0.5	11.25	1.0	13.05	1.63	10.9
0.25	10.95	1.42	11.65	0.25	10.75	0.5	11.9	0.82	11.25
0.125	10.35	0.71	11.5	0.125	10.2	0.25	10.65	0.41	11.3
0.0625	9.9	0.35	11.4	0.0625	9.75	0.125	9.65	0.2	11.2
0.0312	9.2	0.18	11.1	0.0312	9.0	0.0625	8.4	0.1	10.4
0.0156	7.8	0.09	10.1	0.0156	7.5	0.0312	7.35	0.05	9.25
0.0	4.2	0.0							

NH_4Cl		NH_4SCN		$MgSO_4$		$ZnSO_4$*	
Concentration of NH_4Cl.	Alcohol to precipitate.	Concentration of NH_4SCN.	Alcohol to precipitate.	Concentration of $MgSO_4$.	Alcohol to precipitate.	Concentration of $ZNSO_4$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.
2.87	12.95	2.0	13.35	0.5	4.4	0.125	2.7
1.43	11.5	1.0	11.35	0.25	4.75	0.0625	3.0
0.72	10.15	0.5	9.75	0.125	5.05	0.0312	3.05
0.36	8.75	0.25	8.4	0.0625	5.25	0.0156	3.1
0.18	7.6	0.125	7.15	0.0312	5.05	0.0078	3.4
0.09	6.65	0.0625	6.35	0.0156	5.15	0.0039	3.85
0.0	4.4			0.0078	4.9	0.00195	4.0
				0.0	4.5	0.0	4.0

* 2 per cent gelatin A, over night at 30°C. Not strictly comparable to the other experiments in this table.

For all except $ZnSO_4$ (as noted) gelatin B was used (3 per cent) and the tubes stood over night at 26°C. before titrating. For further details see Table II.

tions of the salts, more and more alcohol is needed to precipitate until a maximum is reached. Fig. 2 shows that as the valence of the cation increases, the salt concentration at which the maximum is reached decreases. Valence, however, is not the only

Digitized by Google

factor, $CuCl_2$ being more effective than $CaCl_2$, and $MgCl_2$ less effective. The difference between $CaCl_2$ and $MgCl_2$ is largely due to the fact that acid increases the effect of $CaCl_2$ more than that of $MgCl_2$ and the gelatin used is slighty acid. Similar experiments performed with less acid gelatin show less difference (compare $CaCl_2$ curves in Figs. 2 and 5).

TABLE IV.

Effect of Sodium Salts and Aluminum Sulfate on the Precipitation of Gelatin by Alcohol.

Concentration of salt.	Alcohol to precipitate in			Concentration of salt.	Alcohol to precipitate in		$Al_2(SO_4)_3$.	
	NaCl	$NaNO_3$	Na acetate.		Na_2SO_4	Sodium citrate.	Concentration of $Al_2(SO_4)_3$.	Alcohol to precipitate.
M	cc.	cc.	cc.	M	cc.	cc.	M	cc.
2.0	11.05	10.5	13.3	0.5	3.1	4.4	0.32	Ppt. without alcohol.
1.0	10.1	9.95	10.7	0.25	4.55	6.4		
0.5	9.0	9.0	9.05	0.125	7.1	8.6	0.23	4.85
0.25	8.0	8.0	7.9	0.0625	8.25	9.45	0.16	5.3
0.125	6.85	7.05	6.9	0.0312	8.15	10.05	0.08	5.2
0.0625	6.15	6.05	6.2	0.0156	7.65	10.75	0.04	4.95
0.0	4.4			0.0078	5.8	8.95	0.02	4.89
				0.0039	5.1	5.5	0.01	4.75
3 per cent gelatin B for 15 hrs. at 26°C.				0.00195	4.3	4.45	0.005	5.4
				0.00097		4.3	0.0025	5.2
				0.0	3.9	3.9	0.0012	4.8
				3 per cent gelatin B 15 hrs. at 26°C.			0.0	4.6
							3 per cent gelatin B for 5 hrs. at 26°C.	

Na_2SO_4 and to some extent sodium citrate salt out the gelatin before the true end-point is reached. The white stringy precipitate due to salting out becomes so opaque that in these curves it is taken as the end-point. In $(NH_4)_2SO_4$ in Table V it is possible to titrate through to the usual white homogeneous precipitate. For other details see Table II.

It is also evident from the curves in Fig. 2 that $MnSO_4$ and $CuSO_4$, which combine bivalent cations with a bivalent anion, do not increase the alcohol number at all, but decrease it. This cannot be due entirely to the fact, which Nernst[16] has men-

[16] Nernst, W., Theoretical Chemistry from the Standpoint of Avogadro's Rule and Thermodynamics, London, 3rd edition, 1911, 510.

Digitized by Google

TABLE V.

Effect of Various Monovalent Salts and Salts with One Bivalent Ion on the Precipitation of Gelatin by Alcohol.

KCl		KSCN		LiCl		$(NH_4)_2SO_4$		(NH_4) tartrate.		$CaCl_2$	
Concentration of KCl.	Alcohol to precipitate.	Concentration of KSCN.	Alcohol to precipitate.	Concentration of LiCl.	Alcohol to precipitate.	Concentration of $(NH_4)_2SO_4$.	Alcohol to precipitate.	Concentration of NH_4 tartrate.	Alcohol to precipitate.	Concentration of $CaCl_2$.	Alcohol to precipitate.
M	cc.	M	cc.	M	cc.	M	cc.	M	cc.	M	cc.
0.5	8.65	1.5	11.6	6.65	16.3	1.0	Ppt. without	0.7	Ppt. without	0.5	11.5
0.25	7.45	0.75	10.5	3.22	13.2	0.5	alcohol.	0.55	alcohol.	0.25	11.4
0.125	6.3	0.375	8.9	1.66	11.4	0.25	9.1	0.39	Sets solid.	0.125	11.1
0.0625	5.65	0.19	7.8	0.83	10.1	0.125	9.5 (6)*	0.19	7.4	0.0625	10.1
0.0312	5.05	0.095	6.7	0.415	8.7	0.0625	9.3 (7)	0.09	9.4	0.0312	9.6
0.0156	4.5	0.046	6.0	0.207	7.7	0.0312	9.1	0.045	9.7	0.0156	7.6
0.0078	4.3	0.023	5.4	0.1	6.6	0.0156	8.0	0.0225	9.4	0.0078	5.7
0.0039	4.0	0.012	5.0	0.05	6.1	0.0078	6.25	0.011	9.2	0.0039	5.0
0.0	3.8	0.006	5.0	0.025	5.5	0.0039	5.3	0.0056	9.1	0.00195	4.3
		0.0	4.7	0.012	5.1	0.00195	4.4	0.0028	8.3	0.00097	4.1
				0.006	4.8	0.00097	4.1	0.0014	6.4	0.0	3.9
				0.0	4.4	0.0	4.0	0.0	5.3		
							3.6		4.3		

* Figures in parentheses indicate approximately the point where the "salting out" precipitate appears (see Table IV).

For all experiments in Table V, 3 per cent gelatin B was used and the tubes stood over night at 26°C. before titrating with alcohol.

The difference between the $CaCl_2$ curve given in this table and that given in Table II is due to the greater acidity of the gelatin A used for the latter. For other details see Table II.

Digitized by Google

tioned, that such salts are comparatively little dissociated. If the degree of dissociation were the sole explanation, one would expect $CuSO_4$ to have less effect than $CuCl_2$, but not the opposite effect. In some cases such salts do cause a slight increase in the alcohol number in low concentrations, as for example in the curve for $MgSO_4$ in Fig. 3 and for $Al_2(SO_4)_3$ in Fig. 4. $FeSO_4$

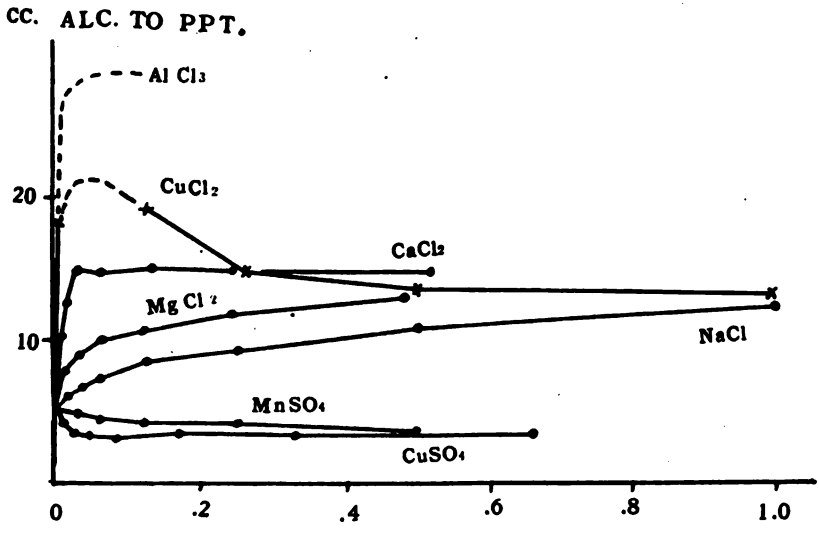


FIG. 2. The ordinates represent the number of cc. of alcohol required to produce an opaque precipitate in 5 cc. of the salt-gelatin mixture; *i.e.*, the alcohol numbers. The abscissæ represent the concentrations of the various salts in the gelatin before titration with alcohol. The effect of the salts increases with the valence of the cation. The dotted lines indicate that no precipitate could be obtained in those concentrations of $AlCl_3$ and $CuCl_2$. Qualitative tests indicate that the $AlCl_3$ curve subsequently comes down a little, like the $CuCl_2$ curve.

also shows a slight increase in the alcohol number in low concentrations.

As proof of the fact that the alcohol number gives a true indication of the effect of salts upon the physical properties of gelatin (in spite of the possibility of repression of ionization of the salts by the alcohol or other interaction) may be mentioned the fact that gelatin sets more readily in solutions of such salts as $MnSO_4$, etc., than in water or solutions of salts with one or

Digitized by Google

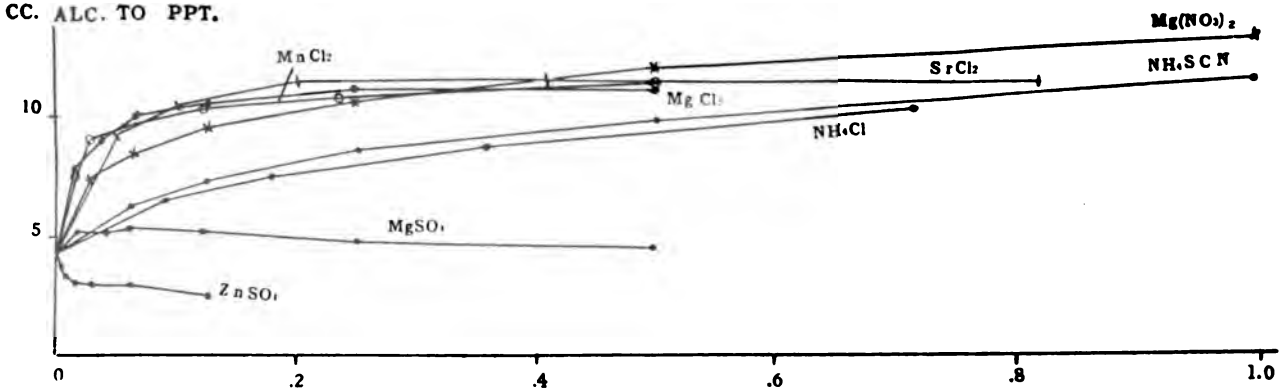


FIG. 3. Alcohol numbers are plotted against concentrations of salts before titration as in Fig. 2. The curves show how clearly three groups of salts are distinguished; *i.e.*, the monovalent salts, salts with one bivalent ion, and salts like $MgSO_4$.

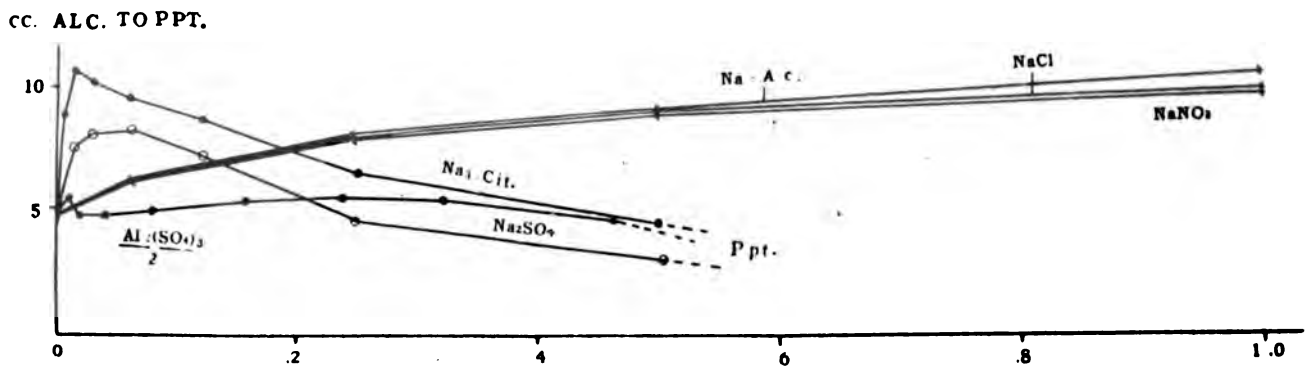


FIG. 4. Alcohol numbers are plotted against concentrations of added salts as in Fig. 2. Sodium citrate has more effect than Na_2SO_4 because it is trivalent or because it has an alkaline reaction, or both. Both these salts "salt out" the gelatin at a concentration of 0.5 M. This accounts for the drop of the curves after the maximum.

Digitized by Google

two monovalent ions. This indicates that one ion of $MnSO_4$ inhibits the dispersing effect of the other on gelatin.

An examination of the curves for Na_2SO_4, $(NH_4)_2SO_4$, ammonium tartrate, and sodium citrate in Figs. 4 and 5 shows that the valence of the anion is also important but that in high concentrations the "salting out" effect of these salts is predominant. The sharp drop of these curves to the point where they precipitate the gelatin without alcohol is due to their strong dehydrating powers. In the case of these salts there are two dis-

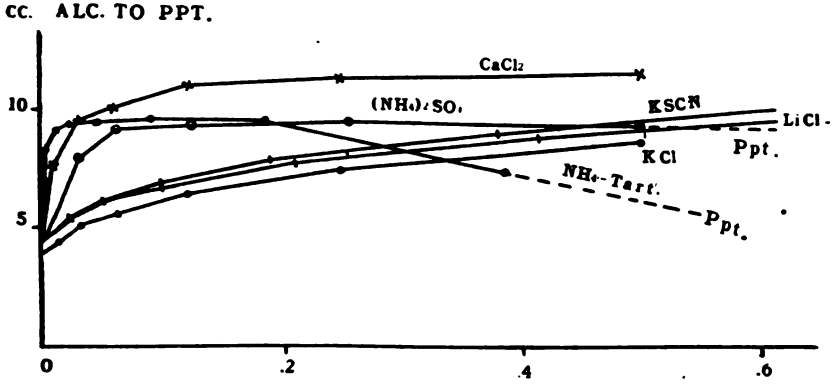


FIG. 5. Alcohol numbers are plotted against concentrations of the salts added to the gelatin as in Fig. 2. This figure shows how distinctly salts with one bivalent ion are distinguished from the monovalent salts in their dispersing action.

tinct end-points to be found in precipitating with alcohol in the higher concentrations. There appears first a stringy precipitate which is followed later by a white homogeneous one. In the case of Na_2SO_4 the stringy precipitate was so opaque that it had to be taken as the end-point, while in the case of $(NH_4)_2SO_4$ it was possible to titrate through to the second precipitate, which had the more usual appearance. This accounts for the fact that the Na_2SO_4 curve drops after the maximum while the $(NH_4)_2SO_4$ curve does not. The first white stringy precipitate is probably due to the "salting out' or dehydrating effect of the SO_4 ion. The second white homogeneous precipitate is regarded as the true end-point.

Digitized by Google

In Fig. 5 the distinctness with which the salts with bivalent ions are separated from the monovalent salts is noteworthy.

The effects of salts upon the alcohol number of gelatin are roughly parallel to their effects on the other properties of gelatin. The temperature of gelation in salt solutions has been measured by Levites[17] and by Pauli and Rona,[18] the swelling of gelatin in salts by Ostwald,[8] Ehrenberg,[11] Pascheles,[19] and Lenk,[20] and its viscosity in salt solutions by von Schroeder.[2] None of these methods of investigation is as simple or as accurate as the alcohol method and the results are correspondingly less complete.

Pauli[21] has investigated the effects of salts upon the temperature of coagulation of egg white and finds them similar to the effects on the coagulation by alcohol. He finds that the bivalent metals are more effective than the monovalent metals in preventing coagulation. The action of salts in dispersing gelatin so that more alcohol is needed for precipitation is also similar to their effects in dispersing globulin. Thus Mellanby[22] has found that the dispersing efficiency of trivalent, bivalent, and monovalent ions were to each other as the squares of their valences, and the efficiency of salts were equal to the sum of the efficiencies of their separate ions. Thus the dispersing efficiencies of NaCl, $CaCl_2$, Na_2SO_4, and $MgSO_4$ are 2, 6, 6, and 8 respectively. This conclusion is borne out by the alcohol experiments except for the fact that salts like $MgSO_4$ have very much less effect on gelatin than NaCl, instead of four times as much.

The effects of salts upon the physical properties of proteins are now known to be accompanied by a combination of the salt with the protein, although Bugarsky and Liebermann[12] were unable to find any evidence of it in the case of NaCl and egg white by their measurements of the freezing point or the electrometric determination of the Cl ion. Hardy[23] has shown by conductivity measurements that NaCl combines with globulin to the

[17] Levites, S. J., *Z. Chem. u. Ind. Kolloide*, 1907–08, ii, 237.
[18] Pauli, W., and Rona, P., *Beitr. chem. Physiol. u. Path.*, 1902, ii, 1
[19] Pascheles, W., *Arch. ges. Physiol.*, 1898, lxxi, 333.
[20] Lenk, E., *Biochem. Z.*, 1916, lxxiii, 15.
[21] Pauli, *Arch. ges. Physiol.*, 1899, lxxviii, 315.
[22] Mellanby, J., *J. Physiol.*, 1905-06, xxxiii, 338.
[23] Hardy, W. B., *J. Physiol.*, 1905-06. xxxiii, 251.

Digitized by Google

extent of 2 per cent, and the writer has found that the same is true for a 1.87 per cent gelatin solution as the following figures indicate:

Concentration of NaCl.	Conductivity depressed.
0.665 M.	1.51 per cent.
0.299 "	1.44 " "
0.145 "	1.66 " "
0.0725 "	1.65 " "

Similar measurements with $CaCl_2$ and Na_2SO_4 both on gelatin and on Witte's peptone have shown that these salts combine more than NaCl.

Pauli[24] has recently made a detailed study of the equilibrium between $AgNO_3$ and gelatin, ox serum proteins, and caseinogen by means of potentiometer and conductivity measurements. He finds that with increasing concentrations of $AgNO_3$ more and more combines with the protein until a maximum is reached. He believes that both ions combine equally. There is evidence that this is not true for the salts of the alkaline earths. Pauli himself states[25] that addition of Ba salts to proteins increases the H ion concentration of the solution as determined by indicators. If this were true it would indicate that the protein combines to a greater extent with Ba than with its anion. Fraenckel[26] has also observed this fact for Ca salts by measurements with a concentration cell. Hardy,[23] furthermore, states that when $CaCl_2$ precipitates globulin, free acid is formed in the solution as determined by methyl orange. Billitzer[27] has found cathodic convection of proteins with the salts of alkaline earths, indicating the presence of a positive charge due to a greater combination of the protein with the cation than with the anion. As further evidence that salts with bivalent cations can deliver a positive charge to colloidal substances may be mentioned the work of Perrin[28] on endosmosis of various solutions through diaphragms of a great variety of substances and the similar work of Briggs,[29]

[24] Pauli, *Biochem. Z.*, 1917, lxxx, 187.
[25] Pauli, *Beitr. chem. Physiol. u. Path.*, 1904, v, 27.
[26] Fraenckel, P., *Z. exp. Path. u. Therap.*, 1905, i, 439.
[27] Billitzer, J., *Z. physikal. Chem.*, 1905, li, 129.
[28] Perrin, J., *J. chim. phys.*, 1904, ii, 601.
[29] Briggs, T. R., *J. Phys. Chem.*, 1917, xxi, 198.

Digitized by Google

des Bancels,[30] von Elissafoff,[31] and others; and the work of Bose[32] and Guillaume[33] on the potential developed when a gelatin-coated wire is suddenly twisted in the solution of an electrolyte. Contrary evidence is found, however, in the fact that neither Chick[34] nor Hardy[23] were able to find any convection of globulins (or of gelatin) in solutions of the alkaline earths. Whatever difference there is, therefore, in the extent to which the cations and anions of the alkaline earths combine with proteins must be too small to detect except by the most delicate measurements of the change in the H ion concentration. The difference would of course be still less in the case of the salts of the alkali metals.

SUMMARY.

1. Acids and alkalies hinder the precipitation of gelatin by alcohol. In the case of strong acids and alkalies this effect passes through a sharp maximum and then decreases.

2. Increasing concentrations of salts tend to hinder the precipitation of gelatin by alcohol until a maximum is reached, trivalent ions being more effective than bivalent, and bivalent more effective than monovalent in this respect.

3. Certain salts (like $MnSO_4$) which combine a bivalent cation with a bivalent anion are exceptions to this rule in that they either assist or only very slightly hinder the precipitation of gelatin by alcohol, the effect of one ion being apparently neutralized by that of the other.

4. Salts like $AlCl_3$ and $CuCl_2$ which are very effective in hindering precipitation by alcohol resemble the strong acids in that their effect passes through a maximum and then decreases.

5. The effect of the sulfates, citrates, and tartrates of the alkali metals also decreases in high concentrations due to their strong "salting out" or dehydrating powers.

[30] des Bancels, J. L., *Compt. rend. Acad.*, 1909, cxlix, 316.
[31] von Elissafoff, G., *Z. physikal. Chem.*, 1912, lxxix, 385.
[32] Bose, J. C., *J. phys. théorique et apliquée*, 1902, series 4, i, 481.
[33] Guillaume, E., *Compt. rend. Acad.*, 1908, cxlvii, 53.
[34] Chick, H., *Biochem. J.*, 1913, vii, 318.

Digitized by Google

FEEDING EXPERIMENTS WITH PEANUTS.

By AMY L. DANIELS and ROSEMARY LOUGHLIN.

(*From the Department of Home Economics, University of Wisconsin, Madison.*)

(Received for publication, December 7, 1917.)

Although, popularly, the peanut (*Arachis hypogœa*) is classed as a nut, it more properly belongs to the grain or forage crop. the "nut" or fruit being the seed of a legume comparable to the bean or pea seed. Its use as human food has been largely limited to peanut butter and confectionery, and little attempt has been made to include it as a regular article of diet. Considerable emphasis, however, has been placed upon its value as food for stock.[1] It forms one of the best forage crops for pigs in the south, while in Europe peanut meal is a much used feed. The good results obtained with it here have suggested that man might profitably add it to his list of staple foods.

In the investigation rats were fed rations consisting of lard, cornstarch, suitable inorganic material, and either ground roasted peanuts or peanut meal. In some cases 2 per cent of butter fat was substituted for an equivalent amount of lard; while in other cases 5 per cent was substituted. One group was given peanut meal to which lard, cornstarch, and butter fat were added, the mineral content being supplied wholly by the peanut meal.

The value of the proteins of the peanut is shown by the performance of animals (Chart I) fed a ration which furnished 18 per cent of protein obtained from peanuts.[2] The rats which were given an insufficient amount of the fat-soluble food accessory failed to grow normally. The addition of 5 per cent of butter fat stimulated growth, and reproduction followed. When very young rats were given this 5 per cent butter fat ration

[1] Henry, W. A., and Morrison, F. B., Feeds and Feeding, Madison, 16th edition, 1916, 178.

[2] The Spanish peanuts used contained 24 per cent protein (N × 6.25).

Digitized by Google

(Chart III, Rats 4, 6, 7, 14, 17, and 18) growth was not satisfactory, death resulting in some cases, apparently due to the fact that the ration contained too much fat, for in order to obtain an 18 per cent protein mixture from peanuts, together with 5 per cent of butter fat, it was necessary to feed a ration containing 38.6 per cent of fat. Young animals on a diet which includes such a high percentage of fat do not seem to thrive. When these young animals were given a ration containing less fat, obtained by decreasing the amount of butter fat from 5 to 2 per cent, better growth resulted. Their curves of growth, however, were somewhat below normal. An adult animal which was given the 2 per cent butter fat ration reproduced and two litters were successfully suckled (Chart I, Rat 12). The litters were small (five and six each) and the young remained undersized as long as they were given the 2 per cent butter fat ration. The addition of 5 per cent of butter fat stimulated growth.

In view of the fact that peanuts contain such a high percentage of fat, it was deemed advisable to continue the investigation with peanut meal which contained less fat. Rations consisting of 67.5 gm. of peanut meal,[3] supplying 18 per cent protein, 10 gm. of lard, 5 gm. of butter fat, 11.4 gm. of cornstarch, and 5.09 gm. of suitable inorganic material proved satisfactory in every way. The curves of growth (Chart II) in all cases were similar to those generally considered to be normal. Reproduction occurred at frequent intervals and three generations were obtained. Good growth was secured also with a ration supplying 15 per cent protein from the peanut meal (Chart II). The proteins of peanuts are comparable to those of the soy bean, since it has been shown that both legumes supply the essential amino-acids in sufficient amounts for normal growth and reproduction, when rations made on the basis of 15 and 18 per cent protein are fed.

That peanuts are lacking in the fat-soluble food accessory has been shown by the behavior of animals which were given rations containing no butter fat, 2 per cent, and 5 per cent of butter fat, respectively. The animals which, immediately following the suckling period, were placed on rations, otherwise adequate but

[3] The peanut meal was furnished by the Capital Grain and Feed Co., Montgomery, Ala., and contained 27.02 per cent protein (N × 6.25).

Digitized by Google

containing no butter fat, grew at about half the normal rate and finally died unless butter fat was given (Chart III). The prompt recovery of the surviving animal (Rat 8) when 5 per cent of butter fat was added indicates that the lack of the fat-soluble food accessory was the inhibiting factor in this diet. The animals receiving the 2 per cent butter fat ration made somewhat better growth than those receiving none. Their curves of growth, however, are below those of normal animals (Chart II). Three females of the group reproduced (Chart I, Rats 6, 12, and 13). Their young were also undersized. Irritable dispositions and marked roughness of coats characterized all the animals of this group. Such conditions indicate malnutrition. The change from the 2 per cent butter fat ration to a 5 per cent ration stimulated growth, and reproduction quickly followed in the animals which had not previously reproduced (Chart III, Rats 7 and 14).

Since all the animals receiving the 5 per cent butter fat ration were normal in every respect, proof is furnished for the presence of a considerable amount of the water-soluble food accessory in the diets. When the peanuts formed 56 per cent of the ration, there was sufficient water-soluble B for the experimental animals.

A comparison of the inorganic content of the peanut and the soy bean shows the peanut to be even poorer than the soy bean in certain respects; namely, calcium, potassium, magnesium, and sulfur.[4] Therefore since the mineral content of the soy bean had been found lacking,[5] various amounts of suitable minerals were added to make the mineral content of the peanut rations similar to that of milk, which previous investigations have demonstrated to be adequate for normal growth of rats. On the other hand, peanut meal,[6] which includes a considerable proportion of hulls, apparently contains sufficient amounts of these necessary inorganic constituents for physiologic well being. Rats fed a mixture of peanut meal furnishing 18 per cent of protein, 5 per cent of butter fat, lard, and cornstarch, to which no minerals were added, grew normally, reproduced, and successfully reared their young (Chart II, Rats 27, 28, and 29).

[4] Forbes, E. B., Beegle, F. M., and Mensching, J. E., *Ohio Agric. Exp. Station, Bull. 255*, 1913.

[5] Daniels, A. L., and Nichols, N. B., *J. Biol. Chem.*, 1917, xxxii, 91.

[6] *Alabama Agric. and Ind. Dept., Bull. 76*, 1916.

Digitized by Google

When we consider the broad areas which may be adopted as fitting habitats of the peanut, and the fact that our food supply tends toward a wider use of the seeds of plants, we do not hesitate to venture the suggestion that the peanut, when rightly supplemented, will form a staple article of the human dietary. Like the soy bean, it needs only to have added suitable inorganic material and the fat-soluble food accessory to make it a complete food.

Ration I.

Peanuts	75.0
Butter	2.0
Starch	16.9
KH_2PO_4	1.5
KCl	1.3
$CaSO_4H_2O$	3.3

Ration II.

Peanuts	75.0
Butter	5.0
Starch	13.9
Inorganic material same as Ration I.	

Ration III.

Peanut meal	67.5
Butter	5.0
Lard	10.0
Starch	11.4
Inorganic material same as Ration I.	

Ration IV.

Peanut meal	56.0
Butter	5.0
Lard	10.0
Starch	22.7
KH_2PO_4	1.7
KCl	1.3
$CaSO_4H_2O$	3.3

Ration V.

Peanut meal	67.5
Butter	5.0
Lard	10.0
Starch	17.5

Ration VI.

Peanuts	75.0
Lard	2.0
Starch	16.9
Inorganic material same as Ration I.	

Digitized by Google

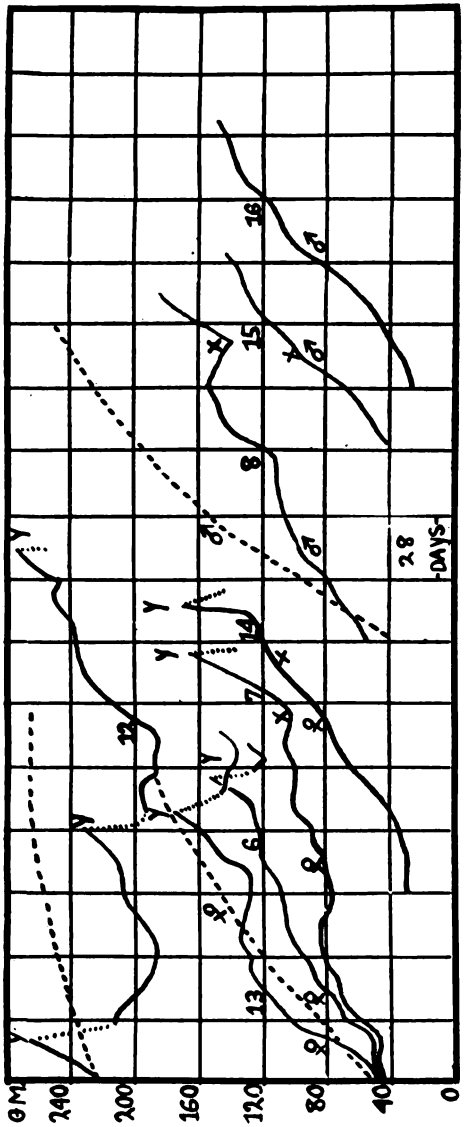


CHART I. Curves of growth of animals fed rations containing 18 per cent of protein, obtained from ground roasted peanuts, a suitable inorganic mixture, and 2 per cent of butter fat (Ration I). Growth was in all cases below normal. Rats 6, 12, and 13 reproduced on this diet and the young, although small, were successfully reared. The addition of 5 per cent of butter fat (Ration II) to the diet of Rats 7, 8, 14, and 15 (indicated by ×) stimulated growth. The broken line indicates the normal curve of growth.

Digitized by Google

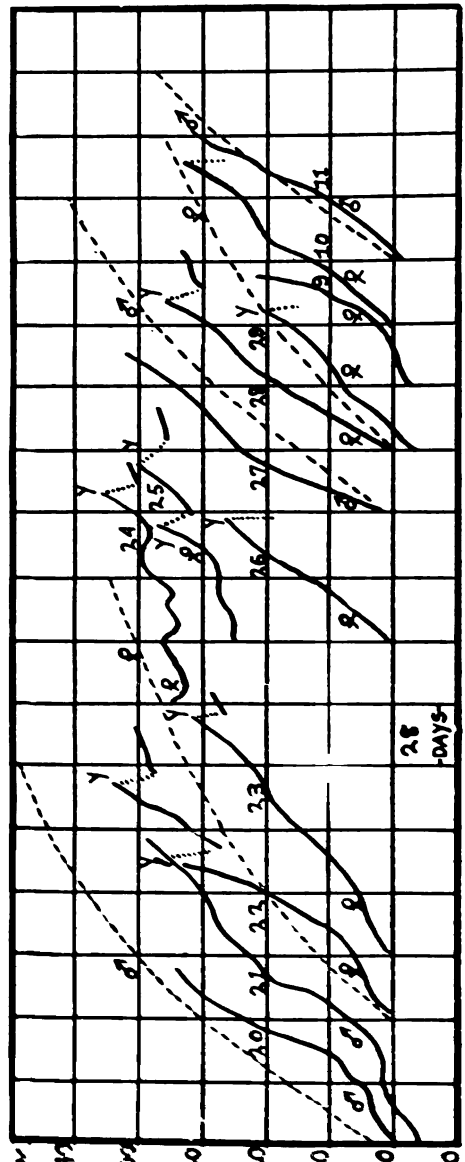


CHART II. The performance of animals (Rats 20 to 26) fed rations containing 18 per cent of protein obtained from peanut meal together with 5 per cent of butter fat and a suitable inorganic mixture (Ration III) demonstrates the efficiency of the proteins of the peanut. Three generations were obtained on this diet. The animals (Rats 9, 10, and 11) on the 15 per cent protein ration (Ration IV) further demonstrate the efficiency of these proteins.

Rats 27, 28, and 29 were given an 18 per cent peanut ration obtained from peanut meal (Ration V), no supplementary inorganic material being included in the diet.

Digitized by Google

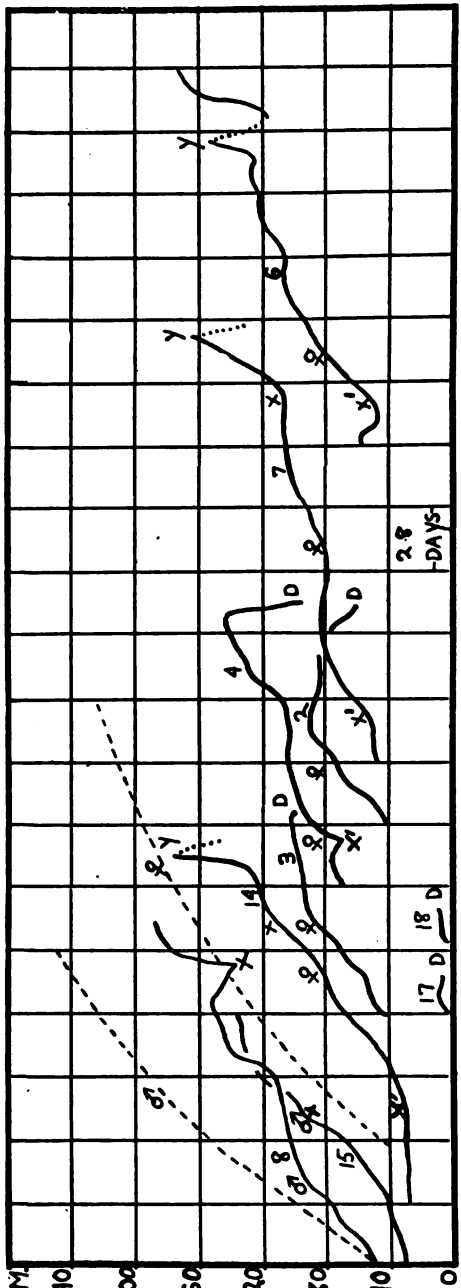


CHART III. Peanuts supply an inadequate amount of the fat-soluble food accessory. These animals (Rats 2 and 3) fed a ration consisting of 18 per cent protein and a suitable salt mixture (Ration VI) grew at about half the normal rate during the 4 months and died. The prompt recovery of Rat 8 when butter fat was added (×) indicates that the fat-soluble accessory was present in too small an amount in this diet. 2 per cent butter fat (Ration I) produced somewhat better growth (Rats 4, 6, 7, 14, and 15). The addition of 5 per cent butter fat to the diet of Rats 7, 14, and 15 stimulated growth.

Very young rats (Nos. 4, 6, 7, 14, 17, and 18) on an 18 per cent protein diet obtained from the peanuts together with 5 per cent butter fat (Ration II) failed to grow. The change to a 2 per cent butter fat ration (×[1]) which supplied less fat content resulted in better growth, 5 per cent butter fat (×) added later further stimulated growth.

Digitized by Google

Digitized by Google

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

IV. THE CAUSES OF FAILURE OF MIXTURES OF SEEDS TO PROMOTE GROWTH IN YOUNG ANIMALS.

By E. V. McCOLLUM and N. SIMMONDS.

(From the Laboratory of Biochemistry of the School of Hygiene and Public Health of the Johns Hopkins University, Baltimore.)

(Received for publication, December 26, 1917.)

Our investigations on the composition of the diet in its relation to growth have made it clear that a food mixture may conform to the most approved standards with respect to its energy and protein content and in affording a considerable variety in flavors, and may be so prepared as to be highly attractive in appearance, and still be of such nature as to fail to nourish properly an animal during growth. Such conditions are easily fulfilled if the diet is restricted to dishes prepared wholly from the seeds of plants or their milling products together with starch, sugars, commercial syrups, vegetable fats, lard, flavoring extracts, and spices.

Our extensive studies of seeds in which a single variety was fed with the addition of single or multiple purified food ingredients, have indicated that the seeds all show a close similarity in their dietary properties. Chemical analysis has revealed pronounced differences in the yields of the various amino-acids obtainable from isolated vegetable proteins. Tests by biological methods have established the fact that corresponding to these differences in the constitution of the proteins are equally great variations in nutritive values. In the preceding paper of this series (1) we have shown that the value of the protein mixture present in each of the more important seeds is without exception much lower than are those of milk. Judging from our studies on the physiological minimum of protein for maintenance in the rat, if milk proteins are assigned the value of 100, oat and millet seed proteins would be assigned a numerical value of

Digitized by Google

about 75, wheat, maize, and rice proteins 50, flaxseed proteins 40, and the proteins of the pea and navy bean about 25. Since the vegetable proteins from various sources exhibit such great variations in their content of the several amino-acids which can be approximately quantitatively estimated, it is reasonable to expect that mixtures of seeds from various sources should in many cases show greatly enhanced values with respect to the biological value of the protein mixture over that of the seeds fed singly as the sole source of protein.

Mixtures of certain seeds should, therefore, on theoretical grounds, be adequate as a source of protein, and the dietary inadequacy of such mixtures should be limited, in the light of our past experience, to a relative shortage of the fat-soluble A and as to the character and amount of the inorganic content. The following table, which was compiled from data by Forbes (2), shows for the seeds, which are of greatest importance as foods in the United States, the great similarity in their content of all inorganic elements except magnesium and calcium. The table also contains data relating to the inorganic content of corn meal and bolted flour.

Mineral Constituents of Seeds. Parts per 100 of Dry Substance.

	Ash.	K	Na	Ca	Mg	P	S	Cl
Wheat	2.08	0.504	0.026	0.041	0.141	0.408	0.009	0.058
Rice, hulled	0.39	0.070	0.016	0.009	0.026	0.091	0.001	0.0004
Oats, rolled	1.83	0.036	0.058	0.097	0.086	0.385	0.005	0.098
Corn	1.55	0.320	0.052	0.011	0.125	0.336	0.160	0.045
Soy beans	3.14	1.162	0.023	0.119	0.169	0.503	0.034	
White beans	3.22	1.177	0.036	0.154	0.148	0.500	0.052	
Cottonseed meal	7.48	1.847		0.236	0.686	1.500	0.037	
Linseed meal	5.84	1.179	0.063	0.351	0.558	0.806	0.076	
Peas		0.940	0.072	0.139	0.150	0.370	0.264	0.034
Barley	1.99	0.270	0.061	0.011	0.150	0.285	0.024	
Patent flour	0.51	0.145	0.003	0.025	0.025	0.111		
Corn meal	0.68	0.163	0.018	0.031	0.061	0.134		
Milk (cow)	5.67	1.160	0.344	0.909	0.088	0.650	0.057	0.791
" (human)	2.42	0.679	0.165	0.288	0.031	0.240	0.018	0.445

Digitized by Google

With the exception of the calcium and magnesium contents the seeds generally resemble each other in most respects in the character of their inorganic content. We have described experiments which show that each of the more important seeds fails to supply an inorganic mixture of a suitable character to support growth (3). The similarity in the inorganic content of the seeds would suggest that mixtures of seeds should be little better with respect to this dietary factor than are the seeds when fed singly. The experiments reported in this paper demonstrate that this is the case.

In the present series of papers in which we are analyzing by biological methods mixtures of foods which are representative of the diets employed in those regions where pellagra is prevalent, we are proceeding from the simple to the complex mixtures determining in each case the exact nature of the dietary deficiency.

The seeds of plants necessarily form a very large part of the diet of man, because when ripe they are sufficiently dry to make them not difficult to keep in a wholesome condition during a long period. Those which are widely employed as food have either when raw or develop during cooking flavors which render them acceptable to the palate. This is in marked contrast to the leaves of plants which with few exceptions contain tannin or other bitter substances which render them unappetizing to man. It was never suspected that the seeds as a class had any common dietary deficiencies until these were made evident by our systematic feeding of each seed with single and multiple additions of the purified food substances, protein, inorganic salts, and the fat-soluble A, in all possible combinations. The biological values of the several food factors contained in the most important seeds, as revealed by this method, have been discussed at length in preceding publications (3).

The fact that the seeds are not complete from the dietetic standpoint has doubtless escaped observation because of the almost universal use of fairly liberal amounts of the flesh of animals and the products of the dairy in this country and in Europe. Moderate amounts of milk supplement the deficiencies of the seeds most satisfactorily and its use forms the greatest factor of safety in our diet.

Digitized by Google

As yet very little is known regarding the supplementary relationships of the proteins of one seed for those of another. One of us (4) has recorded observations on swine which were fed mixtures of two of the cereal grains, nitrogen balances being kept on the animals during a period of growth. These records did not show any marked increase in the value of these protein mixtures for growth as compared with the proteins of the individual grains fed singly. Since at that time the necessity of adding certain salts and a growth-promoting fat was not appreciated, the data obtained cannot be now regarded as satisfactory, and the subject needs further study.

We have in former papers emphasized the principle that it is possible to assign to a food factor a biological value only when the values of all the other factors in the diet are known and remain constant. Thus an animal may be able to grow at the normal rate on a diet, the proteins of which are of such a quality as to scarcely make possible the retention of the necessary amount of nitrogen to support normal growth, if the inorganic content and the two unidentified factors are very satisfactory. On the same food mixture so modified as to make the mineral content less satisfactory, the same protein supply will fail to nourish the animal properly. Distinct benefit can be shown with such rations to follow the improvement of the protein moiety of the diet, when the salt content remains less satisfactory than the optimum. In other words, if one takes an ideal diet as 100 per cent, one factor in that diet can be lowered on the scale to 60 per cent and the animal may make a nearly normal performance, but if two factors are lowered to the value of 70 per cent each, the animal might make a three-fourths normal growth, but if one factor were lowered to 50 and another to 60, signs of malnutrition soon become apparent. As was pointed out in a previous paper (1), one cannot say what is an adequate supply of a dietary factor unless the *biological value* of each of the others is known.

In the past because of the absence of adequate knowledge concerning the number of factors which are essential in the diet and great paucity of data regarding the differences in the quality of the proteins from different sources, students of dietetics have placed great reliance on the energy content of the food mixture as shown by calorimetric studies and the protein content as shown by

Digitized by Google

applying the conventional factor to the nitrogen content, as the criteria by which the ration should be judged. Discussions of the economics of diet have therefore not infrequently, even in very recent times, presented as the main thesis the relative cost of 100 calories of energy and the cost of 100 gm. of protein. As a phase of the discussion of the diet in its relation to pellagra, we wish to illustrate clearly how far these data may fail to indicate the value of a food mixture, especially during growth.

Aron (5), in his studies of the dietaries of the Filipinos, states that the diet of many of the poor laborers consists essentially of about 700 gm. of rice and 250 gm. of fish, the energy value of the fish being equivalent to about 60 gm. of rice. Dairy products are not eaten by these people, and Aron states that the energy value of the fruits and fresh vegetables eaten by his subjects was so small that it might be neglected in his records. Beri-beri is very prevalent where such a monotonous and inadequate diet of rice and fish is eaten.

Chittenden and Underhill (6) have recently described a condition in dogs, which corresponds closely to the syndrome of pellagra in man. They produced this condition by feeding the animals a monotonous diet which consisted of crackers, peas, and cottonseed oil—a diet derived wholly from the seeds of plants. Goldberger (7) has described experiments in which he has produced in man what he and other clinicians familiar with the disease believed to be incipient pellagra, by restricting the diet during 5½ months to dishes prepared from corn meal, corn grits, wheat flour, rice, sugar, starch, syrup, pork fat, sweet potatoes, cabbage, collards, turnip greens, and coffee. We have pointed out (8) that the energy content of the diet was derived to the extent of about 95 per cent from seeds and pork fat. It is with the purpose of emphasizing the impossibility of making an adequate diet from seeds or seed products that we present the data in the accompanying charts. The experimental diets described, illustrate in addition, however, the exact nature of the deficiencies of such seed mixtures. They make clear the necessity both in human nutrition and animal production of supplementing the seeds with either milk or the leafy vegetables in liberal amounts if growth in the young is to be satisfactory, and if the vitality as reflected in longevity and the capacity to produce and rear large

Digitized by Google

numbers of young is to be fostered. Animals, which have been restricted to inadequate and monotonous diets, have in our experience been subject to infections of the lungs and to a condition suggestive of bronchitis, one or both of which constitute the terminal event in the lives of the animals. Xerophthalmia is seen in those animals whose diet lacks an adequate amount of the fat-soluble A and beri-beri when there is a shortage of water-soluble B. Poor condition of the hair, with varying degrees of baldness in some cases, an incrustation of the ears, irritation of the skin, excessive development of warts, especially on the nose and tail, extreme timidity in certain animals brought to a state of malnutrition which in many instances was manifest only in failure to grow, in others by loss of hair and unthrifty appearance, as well as timidity, are common observations in our rat colony, where many types of faulty diets have been employed. Heightened resistance to disease is one of the outstanding features in those groups of our animals whose diets are of such a character as to induce rapid and sustained growth in the young.

Those who select their food solely with a view to economy as respects cost, find in the literature concerning foods the information that the cheapest sources of both energy and protein are the cereal grains, and the legume seeds, peas and beans. Economic conditions are now such that rigid economy is necessary in the purchase of food, especially among city dwellers with small incomes. Unless the public is speedily educated regarding the necessity of employing regularly in the diet either an appropriate amount of milk or of the leafy vegetables, it seems certain that the time is not far distant when dietary errors now becoming common will become a still more important menace to the public health.

The necessity of increasing the content of sodium, chlorine, and calcium in a diet principally derived from seeds before growth can proceed, affords a striking example of the dependence of the growing animal on an appropriately constituted inorganic mixture as a foundation upon which to superimpose a suitable organic ration. The necessity of an adequate supply of those elements which constitute the ash left on incineration of the animal body has been appreciated since the early experiments of Lunin (9) and of Forster (10) with ash-free diets. It

Digitized by Google

seems evident, however, that the efficiency of the mucosa of the alimentary tract, the kidneys, and skin in maintaining an appropriate relationship among the inorganic elements in the blood and tissues by selective absorption and selective excretion has been greatly overestimated. These are important factors of safety, but are not sensitive enough to protect the physiological well-being of the animal under conditions which it may meet in taking a diet exclusively of seeds.

Previous to our solution of the problem of the successful feeding of diets consisting of purified protein, carbohydrates, fats, inorganic salts, and appropriate additions to furnish the two unidentified dietary factors, the problem of determining what constitutes the optimum inorganic content in the diet of a mammal was not possible of solution, since only with natural foods could diets be prepared which were capable of supporting growth. The natural foods show considerable variation in their content of mineral elements, and complicated greatly the analytical control of the composition of the food. The greatest obstacle to the study of the problem lay, however, in the fact that every natural food product contains qualitatively all the mineral elements necessary for the adequate nutrition of an animal. It is impossible to remove these, without at the same time extracting certain organic constituents which might possibly change the dietary value of the substance being studied.

Since we have now perfected the technique of preparing from salt-free materials the organic portion of the ration so as to be highly satisfactory when suitable salt mixtures, prepared from pure reagents, are added, we have thus made possible the investigation of the problems relating to the needs of the mammal for inorganic elements during growth and reproduction. These problems are now occupying our attention.

In a previous paper we have described experiments which showed that the leaf of the plant is very different from the seed from the dietary standpoint. In contrast with the cereal grains the leafy portion of the plant is very rich in the fat-soluble A as well as in the water-soluble B, and is likewise much richer in sodium, chlorine, and calcium than are seeds. These differences in dietary properties go with differences in function. The seed is composed of relatively few functioning cells and much

Digitized by Google

reserve food material. The endosperm is comparable in great measure with a mixture of purified protein, carbohydrate, fats, and inorganic salts. The germ with its high cellular content is relatively richer in both the fat-soluble A and water-soluble B than is the endosperm.

The leaf, on the other hand, is a mosaic of living cells, which is the seat of the great synthetic activities of the growing plant. Corresponding to this difference in function, we have found equally great differences in dietary properties.

SUMMARY OF DATA IN CHARTS.

1. Even with food mixtures derived from two to five seeds we have not found it possible to induce any growth in young rats when the animals were given distilled water to drink, and were therefore, afforded no inorganic salt supply other than what was contained in the seeds. The elements, sodium, chlorine, and calcium, are the only ones which must be added in order to make the seeds complete from the inorganic side. Iodine was given in the drinking water once each week.

2. There is some improvement in the biological values of the proteins of mixtures of seeds in all cases over the values of the proteins of the seeds fed singly. Doubtless in certain cases the values of such mixtures are high, but with simple mixtures of the cereal grains we have not been able to demonstrate a high degree of efficiency as a source of protein in any case.

3. Millet seed and flaxseed are both distinctly better as sources of the fat-soluble A than are the cereal grains, wheat, maize, and oats. Mixtures of seeds containing one of these may, when properly supplemented with calcium, sodium, and chlorine, support animals in apparently good health, and without the appearance of xerophthalmia.

4. Emphasis is laid upon the close similarity of seeds from the dietary standpoint and the danger of deriving the diet almost exclusively from this source.

BIBLIOGRAPHY.

1. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 347.
2. Forbes, E. B., *Ohio Agric. Exp. Station, Bull. 207*, 1909.

Digitized by Google

Digitized by Google

Digitized by Google

Digitized by Google

Digitized by Google

Digitized by Google

Digitized by Google

3. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 615; xxiii, 181; McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105; 1916–17, xxviii, 153; 1917, xxix, 341; xxx, 13.
4. McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 323.
5. Aron, H., *Philippine J. Sc., B*, 1909, iv, 195.
6. Chittenden, R. H., and Underhill, F. P., *Am. J. Physiol.*, 1917, xliv, 13.
7. Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.
8. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 29.
9. Lunin, N., *Z. physiol. Chem.*, 1881, v, 31.
10. Forster, J., *Z. Biol.*, 1873, ix, 297.
11. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 181.

Salt Mixture 185.

	gm.
NaCl	0.173
$MgSO_4$ (anhydrous)	0.266
$NaH_2PO_4H_2O$	0.347
K_2HPO_4	0.954
$CaH_4(PO_4)_2.H_2O$	0.540
Fe citrate	0.118
Ca lactate	1.30

Salt Mixture 500.

	gm.
NaCl	0.5148
$CaCl_2$	0.2569
K_2HPO_4	0.3113
K citrate	0.5562
Ca lactate	2.878

Salt Mixture 318.

	gm.
NaCl	1.40
K_2HPO_4	2.531
K citrate H_2O	0.710
$CaSO_4$	0.578
Ca lactate	7.058

Digitized by Google

Digitized by Google

THYROID HYPERPLASIA AND THE RELATION OF IODINE TO THE HAIRLESS PIG MALADY. I.*

By E. B. HART and H. STEENBOCK.

(From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.)

PLATES 2 AND 3.

(Received for publication, December 17, 1917.)

In our early experimental inquiries[1] on the growth of swine we soon learned that restriction to any one grain and its protein concentrate or a mixture of grains and seed protein concentrates would lead to nutritional failure. Growth would cease and evidences of serious malnutrition appear. It was found that certain supplements to the grains must be made if physiological soundness was to be maintained and growth continued. Among the efficient supplements were tankage, milk, or the leaf and stem portion of plants, such as alfalfa. The plant roughage or tankage was more efficient than milk, the latter ultimately failing as a sole supplement to the grain for perfect nutrition in the proportion used and under strict confinement of the animals. We believe this failure of milk to rest solely on its quantitative relation to the grain mixture and had enough been supplied, success would have followed. With other species of animals and milk as milk powder constituting 10 per cent of the dry matter of a grain ration, successful nutrition has been attained.

After making clear what classes of materials must be used as supplements to the grains for successful growth,—without as yet having finished the problem as to the complete analysis of the factors for successful nutrition contributed by the supplements,—our animals were involved in reproduction. In a number of cases

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373; *Proc. Am. Soc. Animal Production*, 1915, p. 49; *J. Biol. Chem.*, 1916, xxiv, p. xxviii. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239.

Digitized by Google

the young were born dead and hairless or if alive when born hairless, died in a few hours. As it was known to us that this malady existed to a serious extent in certain parts of the northwestern part of this country and also occurred in the state of Wisconsin, our attention was directed to the solution of its cause and remedy. After we were well along in the solution of the problem a paper by Smith[2] appeared from the Montana Agricultural Experiment Station. Smith had reached the conclusion, which our data also support; namely, that the malady was associated with a hyperplasia of the thyroid gland and could be corrected by the use of iodine. He further concluded that the malady was due to a deficiency of iodine in the feed consumed by the brood sows in that locality and believed that if more iodine were fed pregnant animals over large sections of this continent, especially during the winter months, the young that they produced would be more healthy and more vigorous and the large number of weak and defective young animals that are produced annually would be greatly reduced. This last conclusion by Smith is probably open to question, because of the fact that of the vast numbers of farm animals born yearly, but a relatively small proportion are afflicted with those weaknesses that result fatally from a faulty thyroid metabolism and low iodine supply.

Supply of Iodine in Feeds.

The work of Forbes[3] and his associates, and that of Bohn[4] in our own laboratory, show that feeds from all parts of the country are extremely low in their iodine content and with the methods available for its quantitative determination no distinction as to the iodine content of feeds from different localities could be made. It may be a fact that the iodine content of feeds from different regions is very similar, but it is also entirely possible that our analytical methods are still too crude to make the distinctions which may really exist. But while it is true that the iodine content of feeds by the present analytical methods is recorded from

[2] Smith, G. E., *J. Biol. Chem.*, 1917, xxix, 215.

[3] Forbes, E. B., and Beegle, F. M., *Ohio Agric. Exp. Station, Bull.* 296, 1916.

[4] Bohn, R. M., *J. Biol. Chem.*, 1916–17, xxviii, 375.

Digitized by Google

a trace or none to a percentage in the thousandth place, as for beans 0.0002 per cent., wheat 0.0003 per cent, and rarely reaches a thousandth of a per cent, although corn stover showed 0.0012 per cent and celery 0.0016 per cent, it is equally true that the iodine content of the normal thyroid is relatively low. The normal fresh thyroid of a 300 pound pig will weigh about 50 gm., equivalent to about 7.5 gm. of dry matter with 0.2 per cent of iodine or 15 mg. In 100 pounds of feed with 0.0002 per cent of iodine there are 90 mg. of iodine, or six times that of a normal swine thyroid. The rate of metabolism of the thyroid colloid is unknown. The wonderful avidity of thyroid tissue for iodine, as lately demonstrated by Marine and Feiss and Marine and Rogoff,[5] makes it appear more than probable that it is not, at least in all cases, a deficiency of iodine in the feed *per se*, that is the primary cause of thyroid hyperplasia in swine producing hairless pigs, but that the problem is more complex and related to the absorption of iodine from the digestive tract or by the thyroid gland itself.

We have successfully corrected the malady of hairless pig production by the use of potassium iodide, but would hesitate to prescribe potassium iodide as a necessity for normal reproduction for all regions and all animals. The same feeds that produce hairless and dead pigs can be so combined as to produce normal pigs. What the problem needs is a fundamental study of all the factors that may contribute to the final condition of a deranged thyroid gland and its accompanying faulty metabolism. If certain unknown conditions are favorable a sow may obtain from natural feeds the iodine required for both her own and the fetal thyroids, but if unknown and unfavorable conditions prevail the animal may obtain barely enough for her own thyroid activity, but not enough for fetal thyroid development. We have not completed our studies of the problem, but what information we have accumulated is offered as a preliminary report.

[5] Marine, D., and Feiss, H. O., *J. Pharm. and Exp. Therap.*, 1915, vii, 557. Marine, D., and Rogoff, J. M., *ibid.*, 1916, viii, 439.

Digitized by Google

EXPERIMENTAL.

After our early experiments on the growth of swine with grains and grain mixtures had demonstrated the incompleteness of such rations and their disturbing influence on reproduction, attempts were made to determine the amount of alfalfa that must be introduced into the ration to secure a complete cycle of life. With 15 per cent of the air-dried ration, as air-dried alfalfa, good growth and reproduction were secured. This particular ration consisted of 25 parts of corn meal, 25 parts of oat meal, 25 parts of wheat middlings, 10 parts of oil meal, and 15 parts of alfalfa. The pigs were strictly confined to the ration in pens 9 by 12 feet, with shavings as litter. They grew well, but their first litters of pigs were born dead and hairless. The hairless pigs were of normal weight and size and were generally carried a little over the full gestation time,—4 to 7 days. If born alive they lived a few hours after birth, but always died sooner or later. They are characterized by thick pulpy necks and thick skin infiltrated with mucin material. The hair coat may vary and appears to bear a particular relation to their vitality. Where hairless, the skin is smooth, shiny and bald, except for a few hairs around the eyes and nose. In the same litter there may be variation from full hair coats with full vigor to the scanty hair coats and little vitality. The hoofs of hairless pigs are thin walled and undeveloped and the thyroid gland is greatly enlarged.

In our experience the fresh weight of the thyroid from hairless new-born pigs may vary from 0.5 to 3.5 gm., while the fresh weight of the thyroid from the normal new-born pig is rarely over 0.3 gm. It is difficult to state just what a normal thyroid is, for there is considerable variation in weight in the thyroids from apparently normal pigs, but in the 50 to 60 thyroids which we have examined from normal 1 day old pigs the fresh weight varied from 0.160 to 0.800 gm.

The thyroid of sows producing normal pigs also varies in size, and sows of 300 pounds' live weight may have fresh thyroids varying in weights of from 20 to 50 gm. The thyroid of sows producing hairless pigs is likewise large and may weigh 125 gm. in the fresh state. In our experience there is not complete involution, through the administration of potassium iodide, of the

Digitized by Google

thyroid of the mother producing hairless pigs, although it does serve to give the normal sized thyroid to the fetus. As an illustration, a thyroid from a mother that had always produced hairless pigs, but in the last gestation had received potassium iodide, weighed when fresh 112 gm. and was translucent and gelatinous in appearance, indicating a richness in colloid; it contained 0.62 per cent of iodine in the dry matter, which weighed, 33 gm., but the fresh thyroid was of the size of a man's fist while the normal 300 pound pig's thyroid approximates the size of a walnut.

It has already been made clear by the work of Marine[5] and others that the enlarged thyroid, accompanied by the symptoms of myxedema and dermal malnutrition, is a thyroid low in iodine. This enlarged vascular system of the thyroid gland is directly related to an automatic attempt on the part of the organism to furnish a blood of probably lower iodine concentration to the thyroid at a greater rate, in order that this gland may meet the requirements for adequate colloid building. This is exactly the condition of the thyroid in the hairless pig. Its iodine content is either but a trace or none. 1 day old pigs with scanty hair coat contained 0.02 to 0.05 per cent of iodine in the dried thyroid, while the thyroid from the dead and *hairless pigs* contained inestimable quantities of iodine. The iodine content of the *normal* dried thyroid of 1 day old pigs varies from 0.1 to 0.6 per cent. Our own experiments, therefore, confirm in every respect the belief that there is a close relation between the hairless pig malady and the iodine assimilation by either intestine or thyroid of the mother.

After we had produced hairless pigs by the above ration, the same sows were fed during the second gestation period a ration consisting of 33 parts of corn, 33 parts of oats, and 33 parts of alfalfa. On this ration dead and hairless pigs were again produced. With these mothers, once there had been induced serious derangement, through the use of the first ration, of either intestinal absorption or thyroid metabolism, the introduction of a lower protein level and larger amount of roughage did not act as a corrective. Bearing in mind the possibility that alfalfa itself was a contributor to this disturbance the sows were carried through a third gestation on a ration of 33 parts of corn, 33

Digitized by Google

parts of oats, and 33 parts of clover. Again, hairless and dead pigs were born. In the fourth gestation period potassium iodide at the rate of 10 gm. per 100 pounds of feed was given with the above ration,—33 parts of corn, 33 parts of oats, and 33 parts of clover. On this ration normal, vigorous and healthy offspring were produced. The thyroids from the pigs born in the third gestation were large, weighed 2 to 3 gm. fresh, and contained no iodine. In the fourth gestation, the fresh thyroids of 1 day old pigs weighed from 0.250 to 0.4 gm. and contained 0.3 to 0.4 per cent of iodine in the dry material.

The proportion of potassium iodide fed was arbitrarily chosen and possibly less would adequately correct the trouble. Marine and Kimball[6] say that 1 mg. of iodine given weekly by mouth is ample to prevent goiter in dogs. The rate of 10 gm. per 100 pounds of feed is equivalent to approximately 0.016 per cent of iodine, which is much more than is contained in natural feeds and which can give successful reproduction. If, however, it should be found wise to insure our herds of breeding swine against this trouble, it can be done at the present price of potassium iodide (7 dollars per kilo) for 30 cents per head and used during the full gestation period.

After observing these first results with 15 per cent of alfalfa in the ration and a high protein intake during the growing period, we believed that it would be a simple matter to produce enlarged thyroids and hairless pigs by feeding almost any combination of food materials, particularly from plant sources. Consequently sows of 30 to 50 pounds' weight were started on rations made up of 75 parts of corn and 25 parts of alfalfa; 75 parts of rolled oats and 25 parts of alfalfa; 75 parts of wheat and 25 parts of alfalfa. These animals grew well, although slightly below the normal rate. They were fed natural water as had been the case in all the other experiments, but were strictly confined. The first litters of all of these sows on these rations were strong and vigorous. There was not a scanty hair coat in any of the litters or an enlarged thyroid among those examined. The thyroids weighed from 0.100 to 0.300 gm. fresh and contained 0.25 to 0.40 per cent of iodine in the dry material. The feeds were from the same

[6] Marine, D., and Kimball, O. P., *J. Lab. and Clin. Med.*, 1917, iii, 40.

Digitized by Google

sources as in the previous experiments and as far as analytical methods could show, contained no more iodine than those used in our first ration.

The difference between the two types of rations was not in the iodine content, but the percentage of protein and the percentage of roughage. The first ration contained 14 to 15 per cent of protein and the second about 10 per cent. The first ration contained but 15 per cent of alfalfa and the second 25 per cent.

It has been believed for some time that the thyroid gland serves as a detoxicating agent and that the hyperplasia results from stimulation to the gland to produce more and more colloid for purposes of detoxication. Such an hypothesis must assume that the iodine compound produced by the thyroid is, under conditions of high toxicity, either eliminated from the blood stream at a more rapid rate than normally, or is insoluble in the blood stream and cannot transfuse the placental wall. It assumes that there is just as much iodine coming into the blood stream through the intestinal walls as under normal conditions. If this is the case, however, it is difficult to comprehend why there should be a deficiency of iodine for the fetal thyroid unless we assume, as stated above, that there is a more rapid elimination of iodine compounds or the formation of iodine compounds insoluble in the blood.

The fact that our first ration was high in protein and low in roughage would suggest an alternate hypothesis. Such a ration would lead to more or less intestinal putrefaction with the accompanying conditions of faulty assimilation. A faulty assimilation of the iodine compounds in the food would lower the iodine supply in the blood. This would lead to the hyperplasia of the thyroid gland of the mother as well as that of the fetus. Or it is possible that the selective absorption of iodine, instead of being wholly referred to the intestine, may be referred directly to the thyroid gland. Toxic substances produced through putrefaction in the intestine or coming directly from the food may, after absorption into the blood, cause a derangement of the iodine-absorbing mechanism of the thyroid gland; only then as the mass of iodine in the blood is further increased by the administration of iodine, can normal absorption by the thyroid gland

Digitized by Google

be reestablished. This condition could apply to the fetus as well as to the mother. This hypothesis would not preclude the possibility of a goitrous condition due to an actual deficiency of iodine in the food material in certain regions, if it really existed. It would, however, explain the differences observed in the effect of the two types of rations mentioned above and, further, explain the sporadic occurrence of the hairless pig malady. This disease occurs more often in gilts than in old sows. It is more likely to occur in the spring and disappear with the fall litters. On one farm in Wisconsin fifteen from a herd of twenty brood sows produced hairless pigs in the spring of 1917. These sows received a ration of flour middlings, ground oats, skim milk, oil meal, and some ear corn. The slop for the greater part of the winter contained mostly skim milk. This is a high protein ration and probably somewhat constipating. These same sows on summer pasture, with more exercise and roughage farrowed large, healthy litters. The high protein levels fed gilts, the lessened exercise, and more constipating foods of winter are exactly the conditions accompanying the greater prevalence of this malady; and these are the more likely conditions leading to a disturbed assimilation or absorption of iodine.

The hypothesis that the toxicity comes directly from the food materials, as distinct from having its origin in intestinal putrefaction, and leads to a deranged thyroid metabolism is not outside the pale of possibilities. Certain saponins, as digitalin, and toxic substances, as ricin and abrin, are said to produce thyroid hyperplasia. The fact that the hairless pig malady in the northwest is in some cases sharply limited to certain areas, but that within these areas there are unaffected spots, as reported by Smith,[2] strongly suggests the possibility that toxic plants may be concerned in the production of a faulty thyroid metabolism and hairless pigs in some of these localities.

Recently Burget[7] has attempted to produce thyroid hyperplasia in rats experimentally. In this he succeeded through the instrumentality of unsanitary conditions and high protein diets. Further he presents evidence to show that the enlarged thyroid is not produced by specific infection, a view which is in harmony

[7] Burget, G. E., *Am. J. Physiol.*, 1917, xliv, 492.

Digitized by Google

with our own observations. One of two sows, reared on the same ration and in the same pen, produced hairless pigs and the other produced sound and vigorous offspring. These results can be ascribed to constitutional differences, inherited factors which always display themselves with any group of animals. The ration that is near the border line of deficiency in iodine and at the same time is slightly out of balance in some other respect may well affect some individuals more than others.

The sows that had been reared on grain and alfalfa (75 to 25 parts) with successful reproduction, were next taken to higher protein levels and lower roughage,—a ration of 15 parts of alfalfa, 45 parts of corn, 15 parts of oil meal, and 25 parts of middlings. Here there has also been successful reproduction. The young pigs were well haired, and the thyroids normal and with normal iodine content. It appears probable, therefore, that the disturbances to thyroid metabolism are more likely to be developed during the period of most rapid growth of this species than after maturity, but final conclusions in this respect cannot as yet be made.

SUMMARY.

A brief summary of our experience with the hairless pig malady supports the view that it is occasioned by a low iodine assimilation by either intestine or thyroid, resulting in a goitrous condition in both mother and young. This condition interferes more severely with fetal development than with the normal maintenance of the mother.

It appears to be produced by rations with high protein levels and low laxative effects, with the accompanying condition of lack of exercise and unclean surroundings. Young sows exposed to such conditions are more prone to develop thyroid enlargement than are mature sows. We recognize, of course, that there is the possibility that some rations from certain regions are naturally so low in iodine as to make the scarcity of iodine the direct cause.

We have found it possible to grow sows to maturity on natural feeding materials grown in southern Wisconsin, but of relatively low protein content and good laxative properties, with the production of normal offspring. On the other hand, these same

Digitized by Google

feeds, combined in different proportions and fortified with protein concentrates lead to the production of hairless pigs.

We are of the opinion, for the present at least, that we have not reached the stage where it is wise to advocate the *general* use of iodine in the feed of all brood sows; that the conditions leading to the production of hairless pigs should first be analyzed fully in order that we may acquire a complete understanding of the reasons for the successful rearing of sows intended for breeding purposes through the use of natural materials, unfortified with potassium iodide.

However, in regions and on farms where hairless pig production is endemic or persistent in character the direct use of iodides should be made.

In the historical, as well as the practical phases of goiter prevention some observations by Marine are of interest. He says:[4]

"The first instance of preventing goiter on a large scale was accidental and in connection with the sheep raising industry of Michigan. Prior to the discovery of salt deposits around the Great Lakes, the future of the industry seemed hopeless, but with the development of the salt industry and its use by the sheep growers, goiter rapidly decreased. The salt contains appreciable quantities of both bromine and iodine. . . . . The second instance of goiter prevention on a large scale was in brook trout. Some years ago the development of goiter in artificially raised members of the salmon family became alarming and many plants were abandoned on account of the disease. After considerable work, which led to the conclusion that the disease was simple goiter, we were able to completely prevent the disease in several hatcheries, by the use of very small amounts of tincture of iodine added to the water."

It is therefore to be expected, that with the elucidation of the cause of the hairless pig malady, a prompt remedy, when necessary, can be applied.

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. Dead and hairless pigs. Note the thick necks, indicating a goitrous condition. The mother of these pigs had been reared on a high protein and low roughage diet, consisting of 15 parts of alfalfa, 25 of corn, 25 of oats, 25 of middlings, and 10 of oil meal, and kept under strict confinement. She had produced two litters of hairless pigs before being changed to a ration of 33 parts of clover, 33 of corn, and 33 of oats, on which the above pigs were produced.

Digitized by Google

FIG. 1.

(Hart and Steenbock: Hairless pig malady. I)

Digitized by Google

Digitized by Google

FIG. 2.

FIG. 3.

(Hart and Steenbock: Hairless pig malady. I.)

Digitized by Google

Digitized by Google

PLATE 3.

FIG. 2. The same sow that produced the pigs shown in Fig. 1. On the same ration,—33 parts of corn, 33 of oats, 33 of clover,—but fortified with 10 gm. of potassium iodide per 100 pounds of feed, a normal litter was produced.

FIG. 3. Reared on a ration of low protein and comparatively high roughage content, 75 parts of corn, 25 of alfalfa. The iodine content of this ration was not unlike that of the ration which produced the results shown in Fig. 1; the two rations, however, differed in their protein and roughage content and in this case the low protein diet was consumed during the life of the animal, including the growing period.

Digitized by Google

Digitized by Google

ON THE ESTIMATION OF AMINO-ACID NITROGEN IN THE BLOOD.

By SEIZABURO OKADA.

(From the Medical Clinic, Imperial University of Tokyo.)

(Received for publication, December 13, 1917.)

Since the publication of the nitrous acid method for the determination of amino-acid nitrogen by Van Slyke[1] in 1909, the literature upon the occurrence of amino-acids in blood and tissues has grown rapidly. Various procedures have been proposed by different authors for the preliminary removal of protein necessary to the analysis. For the most part the original method of Van Slyke and Meyer[2] has been followed, in which the blood proteins are removed by precipitation with nine or ten volumes of 95 per cent alcohol.

Recent investigations by Folin and Denis,[3] Greenwald,[4] and Bock,[5] however, have shown that the use of alcohol is not effective owing to the fact that amino-acids do not completely escape precipitation by alcohol. Folin and Denis state that certain nitrogenous substances such as creatine, asparagine, and tyrosine added to the blood could not be recovered quantitatively after precipitation with methyl alcohol. Greenwald found that methyl alcohol filtrates contained only a little over half of the amino-acids added to the blood. After a number of experiments with several protein precipitants, he found that trichloroacetic acid closely approximated the ideal precipitant. Nine volumes of 2.5 per cent solution to one volume of blood were adopted and the filtrates treated with a little kaolin. It was found, in every case, that the trichloroacetic acid filtrate contained all the added amino-acid nitrogen. The liquid remains clear after the addition of picric acid or of potassium mercuric iodide, giving no biuret reaction. Bock, as a result of his study on this question, concluded that the trichloroacetic acid procedure gives satisfactory results,

[1] Van Slyke, D. D., *Proc. Soc. Exp. Biol. and Med.*, 1909–10, vii, 43; *J. Biol. Chem.*, 1911, ix, 185.

[2] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

[4] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

[5] Bock, J. C., *J. Biol. Chem.*, 1916–17, xxviii, 357.

Digitized by Google

but is somewhat troublesome in certain stages of the manipulation. Filtration is very slow, and the alternative process of centrifuging such large volumes (300 to 500 cc.) is not always convenient. To avoid this difficulty, he recommended heat coagulation followed by trichloroacetic acid precipitation.

From the standpoint of accuracy the procedures of Greenwald and Bock are both satisfactory, but some difficulties still remain, chiefly the removal of the trichloroacetic acid after precipitation of protein. As suggested by Bock, if the trichloroacetic acid is not entirely removed it enters into some combination with the amino-acids which prevents their quantitative reaction in the Van Slyke method. Vacuum evaporation does not suffice for this purpose and Bock's procedure of direct evaporation until the color change of the alizarin indicator is not convenient, for, as the solution reaches dryness, frothing and bumping often occur; further, the operation requires from 30 to 50 minutes. After making alkaline to remove ammonia and acidifying again, it is difficult to get clear solutions. To avoid this difficulty, I sought to obtain a method in which it is not necessary to remove the protein precipitant or in which it could be removed without much difficulty. Kaolin in large quantities was found appropriate for this purpose. The preliminary procedure was carried out according to Bock. The details of the whole procedure are as follows.

A measured volume of blood (30 to 50 cc.) is treated with 0.4 gm. of ground soy bean dissolved in 2 to 3 cc. of water and 1 cc. of a 3 per cent solution of NaH_2PO_4, and allowed to stand at room temperature for 30 minutes. The urea contained in the blood is converted into ammonia which is removed afterwards. One volume of this blood is slowly added to five volumes of boiling 0.01 N acetic acid in a casserole and boiled with stirring for half a minute. The same amount of boiling water is then added and the mixture is boiled with stirring for a moment, giving a clear and easily filterable precipitate. This is filtered hot through a folded filter and the casserole washed three times with 30 to 50 cc. portions of boiling water. The filtrate is rapidly concentrated over a free flame in a casserole. The residue is transferred quantitatively to a graduated cylinder or flask, making the volume nearly that of the original volume of the blood. After adding 0.1 N

Digitized by Google

acetic acid, the solution is made exactly one and a half times the volume of the blood taken. This is then shaken with kaolin (20 gm. per 100 cc.) and immediately filtered through a folded filter. The first small portion of the filtrate is usually cloudy and should be refiltered with the same filter. When in a great hurry, it is recommended to centrifuge before filtration, but in this case it is necessary to repeat the procedure again to get clear solutions. The filtrate is thus quite clear, giving no turbidity or precipitation with trichloroacetic acid or with picric acid even after standing for 24 hours, and no biuret reaction even after concentrating as far as possible. An aliquot part (30 to 60 cc.) of the filtrate is made distinctly alkaline with 1.0 N potassium hydroxide (1 to 2 cc.), the ammonia removed by boiling 2 minutes, the solution made distinctly acid with acetic acid, and concentrated to the smallest possible volume; it is then transferred to a small, accurately graduated test-tube and made to definite volume (5 to 10 cc.). The heat-kaolin method gives filtrates which rarely exhibit any tendency to froth, when shaken in the deaminizing bulb. Should frothing occur, treat again with a small portion of kaolin and measure the filtrate directly. Caprylic alcohol, as recommended for other methods, is entirely superfluous by this method.

With regard to the efficacy of the method in the recovery of added amino-acids, I first determined whether kaolin would absorb amino-acids. The following results indicate that kaolin even in a large amount does not absorb amino-acids at all (Table I).

To ascertain whether the thorough manipulation described involves losses of amino-acids, the following experiments were performed.

10 cc. of leucine solution, containing 5.42 mg. of amino nitrogen were added to 33 cc. of rabbit blood, which contained 3.81 mg. of amino nitrogen. The total amino nitrogen found was 9.07 mg., or a recovery of 5.26 mg. of amino nitrogen from the leucine. Similarly, of 13.70 mg. of amino nitrogen in 10 cc. of alanine solution, after addition to 30 cc. of blood, 13.42 mg. were recovered, and from 40 cc. of blood, 13.30 mg. were recovered. 10.18 mg. of amino nitrogen were recovered when 10.35 mg. in 10 cc. solution of amino-acids from casein were added to 30 cc. of blood.

Folin and Denis state that, "All reagents involving heating

Digitized by Google

TABLE I.*

Recovery of the Nitrogen of Amino-Acids. Amino-Acid Nitrogen per 10 Cc. of Solution.

Material.	Amount of kaolin added.	Amino-acid nitrogen.
	gm.	mg.
Tyrosine	0	1.67
"	1, 2, 3, or 4	1.67
Alanine	0	13.70
"	2, 3, or 4	13.70
Amino-acids from casein	0	10.35
" " "	2 or 4	10.35

* For the pure amino-acids I am indebted to Professor M. Kumagawa and his staff. Amino-acids from casein were obtained by hydrolyzing pure casein by boiling with 25 per cent hydrochloric acid for 30 hours. The liquid was neutralized with sodium hydroxide and filtered. The filtrate was treated with animal charcoal and filtered. After the solution was found entirely free from protein, it was diluted to a convenient concentration of amino-acid nitrogen and treated by the Van Slyke method. As ammonia is found in the hydrolysis of casein, it was removed before the determination of the amino-acid nitrogen.

are useless, because by heat (half an hour in a water bath) the nitrogen of normal blood filtrates may be increased to twice the real value." No figures are offered confirming this statement. Bock attempted a comparison between the direct trichloroacetic acid precipitation and the heat coagulation followed by the trichloroacetic acid precipitation, and has shown that the latter procedure gives even slightly lower results than the former. As kaolin could not be used directly for the blood protein precipitant, a comparison between the direct kaolin precipitation and the heat coagulation followed by kaolin precipitation on blood plasma was carried out. Direct kaolin precipitation was carried out as follows: The blood plasma, diluted ten to twelve times with distilled water, was acidified with 2 to 4 cc. of glacial acetic acid, 40 gm. of kaolin per 100 cc. of diluted solution were added, then the mixture was filtered with suction. After the filtrate was found entirely free from protein, an aliquot was taken and evaporated to a small volume. For the determination of amino-acids, urea and ammonia were removed by usual procedure. Tables II and III show no substantial difference between the procedures.

Digitized by Google

TABLE II.

Non-Protein Nitrogen per 100 Cc. of Blood Plasma (Determined by Kjeldahl.)

Material.	Kaolin precipitation.	Heat coagulation followed by kaolin precipitation.
	mg.	*mg.*
Beef plasma (oxalated).....	10.08	10.51
" " "	9.67	9.11
" " "	18.22	18.56
" " "	18.07	18.22

TABLE III.

Amino-Acid Nitrogen per 100 Cc. of Blood Plasma.

Material.	Kaolin precipitation.	Heat coagulation followed by kaolin precipitation.
	mg.	*mg.*
Beef plasma (oxalated).....	6.42	6.36
" " "	5.54	5.64
" " "	3.80	3.97
" " "	5.26	5.24
" " "	4.97	5.06

A comparison of the heat coagulation followed by trichloroacetic acid[5] and the heat coagulation followed by kaolin precipitation shows that there is no appreciable difference between these two methods (Table IV).

TABLE IV.

Comparison between the Heat-Trichloroacetic Acid Method and the Heat-Kaolin Precipitation. Non-Protein Nitrogen per 100 Cc. of Blood (Determined by Kjeldahl).

Material.	Heat-trichloroacetic acid.	Heat-kaolin.
	mg.	*mg.*
Beef blood (oxalated).....	14.29	14.29
Dog " "	23.78	23.54
" " "	23.88	24.18
" " "	18.21	18.21
" " "	25.50	25.50
" " "	26.90	26.45

Digitized by Google

TABLE V.

Comparison between the Heat-Trichloroacetic Acid and the Heat-Kaolin Precipitation. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Heat-trichloroacetic acid.	Heat-kaolin.
	mg.	*mg.*
Rabbit blood (oxalated).....	10.49	9.82
" " "	11.68	11.68
Sheep " "	6.70	6.70
Rabbit " "	11.57	11.57
" " "	10.70	10.87
Beef " "	7.42	7.42

Alcohol precipitation has shown more or less lower values as was expected from the results of previous investigations.

TABLE VI.

Comparison between the Heat-Kaolin and Methyl Alcohol Method. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Heat-kaolin.	Methyl alcohol.
	mg.	*mg.*
Rabbit blood (oxalated).....	9.72	5.89
" " "	10.55	8.68
" " "	10.67	9.53
" " "	9.17	8.99
" " "	9.81	8.49

TABLE VII.

Comparison between the Heat-Kaolin and Ethyl Alcohol Method. Amino-Acid Nitrogen per 100 Cc. of Blood.

Material.	Heat-kaolin.	95 per cent alcohol.
	mg.	*mg.*
Dog blood (oxalated).....	8.06	5.05
Rabbit " "	7.97	5.78
" " "	7.06	5.57
" " "	11.33	7.27
Dog " "	6.57	3.84
" " "	9.89	5.17

Digitized by Google

SUMMARY.

The use of methyl or ethyl alcohol as a precipitant for proteins in the determination of non-protein nitrogen or amino-acid nitrogen in blood is undesirable.

The methods of Greenwald and of Bock have been found serviceable for amino-acid nitrogen determination, but the procedures are somewhat troublesome.

A method is proposed (the heat-kaolin method) which has been found accurate, less troublesome, saving in time, and economical.

In conclusion I wish to acknowledge my indebtedness to Professor T. Aoyama for his generosity in allowing me to carry out this work in his laboratory and for his encouragement.

Digitized by Google

Digitized by Google

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

I. HIGH LEVELS OF CO_2 AND ALKALI.

By YANDELL HENDERSON and H. W. HAGGARD.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, December 31, 1917.)

The balance between acids and alkalies has come to be recognized as one of the most important of the equilibria of the living body. It is so important in fact that it appears never to be considerably altered except when the organism is practically moribund.

The two modes of estimating the degree of equilibrium which, at the present time, appear to be most interesting are by measurement of the CO_2 content of the alveolar air and of the CO_2 capacity or "alkaline reserve" of the blood (Van Slyke). The view now prevailing is that in "acidosis" the accumulation of non-volatile acids in the body neutralizes the alkalies and causes a reduction in both measurements.

In the facts and conclusions which follow we do not question the essential correctness of this general conception in conditions of true acidosis.[*] We do however attempt to show that many conditions simulating acidosis are really due to disturbance of another factor which exerts a powerful influence upon the CO_2 capacity of the blood, and which may lead to an equilibrium at a new level. This factor is respiration. Abnormal breathing may even produce a disturbance such that equilibrium cannot be spontaneously restored. It may thus produce death.

Recent papers by Scott,[1] Peters,[2] and others have recognized certain aspects of this factor. General recognition of its power will profoundly modify the present crassly mechanistic theoreti-

[1] Scott, R. W., *Am. J. Physiol.*, 1917, xliv, 196.

[2] Peters, J. P., Jr., *Am. J. Physiol.*, 1917, xliii, 113; xliv, 84.

Digitized by Google

cal conceptions in this field, and will, we believe, find wide reaching practical applications.

In brief, the experiments to be described in this series of papers seem to indicate that when in an otherwise normal organism respiration is stimulated to excessive vigor by pain, by a drug, *e.g.*, ether (or probably by any condition which lowers the threshold and increases the sensitivity of the respiratory center), the blowing off of carbon dioxide produces a reduction in the CO_2 content of the blood. This condition very soon leads to (and in respect to C_H is probably in part compensated by) a reduction also in the CO_2 capacity (alkaline reserve) of the blood. Apparently alkali passes out of the blood into the tissues. Thus the acid-base equilibrium or CO_2: alkali balance of the blood is restored at a level much below normal, and a condition simulating, but in certain respects markedly distinct from true acidosis results. In fact, until thus compensated, the condition is a sort of low CO_2 alkalosis. It may be conveniently designated acapnia,[3] although the term thus applied has a significance much broader than heretofore. It indicates a blood low not only in CO_2 (H_2CO_3), but also in sodium bicarbonate. Unlike a true acidosis the total alkali in the body remains, so far as we can judge, unaltered; it is merely abnormally distributed.

On the other hand when the respiratory center is depressed *e.g.*, by morphine, or when by the administration of high concentrations of CO_2 in the air breathed, the alveolar CO_2 is raised, the CO_2 content of the blood is also raised. This causes (and in respect to the C_H of the blood is probably compensated by) an increase in the CO_2 capacity or "alkaline reserve" of the blood, due apparently to the passage of alkali from the tissues into the blood. Thus a pseudoalkalosis or, if one thinks only in terms of C_H, a CO_2 acidosis, is produced. It may conveniently be termed hypercapnia: both the CO_2 and the sodium bicarbonate in the blood are above normal. Probably the total alkali in the body is unaltered. It is merely redistributed.

Either of these conditions, when once induced, shows a distinct tendency to be maintained. Any rapid variation from the new

[3] Henderson, Y., *Am. J. Physiol.*, 1908, xxi, 126; 1908–09, xxiii, 345; 1909, xxiv, 66; 1909–10, xxv, 310, 385. Henderson, Y., and Scarbrough, M. McR., *ibid.*, 1910, xxvi, 260. Henderson, *ibid.*, 1910–11, xxvii, 152.

Digitized by Google

level of CO_2 and alkali back toward the normal level is resisted by the action of the respiratory center or allowed to develop only gradually.

In this paper we shall give the experiments in which a high level of CO_2 and alkali, or hypercapnia, was induced. In succeeding papers experiments producing low levels of CO_2, or acapnia, will be presented.

Theoretical.

Attention at present is fixed so exclusively upon C_H that we must specifically point out that actually this "physiological constant" only varies imperceptibly. Even these slight variations induce powerful counteracting efforts. Through these efforts nearly normal equilibrium in respect to C_H is attained at highly abnormal levels of CO_2: alkali. Thus, as we see it, interest should now shift, at least for the physiologist, from C_H and the physicochemical equilibrium to these levels of CO_2:alkali and the physiological equilibrium.

The physicochemical equilibrium (L. J. Henderson) is approximately the balance of CO_2 against $NaHCO_3$ to maintain $\overset{+}{H}$, that is, C_H, in the expression:

Pulmonary air.	Blood.
CO_2 $\rightleftharpoons$	Dissolved $CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons \overset{+}{H} + \overset{-}{HCO_3}$
	$\overset{+}{Na} + \overset{-}{HCO_3} \rightleftharpoons NaHCO_3$

On the other hand the physiological equilibrium and the respiratory activity by which any great and sudden changes in C_H are prevented may be expressed, for example, at three levels of CO_2 and alkali as shown in the following table.

The numerator (H_2CO_3) is always proportional to the alveolar CO_2 because the amount of CO_2 dissolved (not combined with alkali) is determined by the tension of CO_2 in the alveolar air (law of Henry). The alveolar CO_2 is of course always inversely proportional to the pulmonary ventilation per unit mass of CO_2 eliminated.

Digitized by Google

Blood Plasma.		C_H	Respiration.		Condition.
CO_2 in form of			CO_2 in alveolar air.	Pulmonary ventilation in cc. of air per 5.5 cc. CO_2 eliminated.	
$H_2CO_3+NaHCO_3$	$\frac{H_2CO_3}{NaHCO_3}$				
vol. per cent	*vol. per cent*		*per cent*	*cc.*	
63	3:60	Normal.	5.4	100	Normal level of CO_2: alkali.
42	2:40	"	3.6	150	Low level of CO_2: alkali.
84	4:80	"	7.2	75	High level of CO_2: alkali.

The expression "level of CO_2 and alkali" does not mean the proportion of CO_2 to alkali but the quantity of both; that is, their sum. Thus when in the plasma the proportion of H_2CO_3:$NaHCO_3$ is 3:60 the level of the equilibrium (C_H) is normal or nearly normal at 63. When it is 2:40 the equilibrium is at the low level of 42, while 4:80 is at the high level of 84. In all these cases the proportion of H_2CO_3:$NaHCO_3$ is equal to 1:20 and the C_H may (theoretically) be the same; that is, normal (Van Slyke and Cullen[4]). The breathing to maintain this equilibrium must, for a definite CO_2 elimination, be 50 per cent greater than normal at the low level and 25 per cent less than normal at the high level. Whenever the proportion is forced away from 1:20 (or its equivalents in respect to C_H 3:60, 4:80, 2:40) the system seeks by physiological means to reattain equilibrium, in as nearly the normal C_H as possible, at a new level. If the proportion is more than 1:20 the C_H is above normal and if less than 1:20 it is below normal (cf. Peters).[2]

These figures apply to plasma, in which, at least for theoretical discussion, the conditions are comparatively simple. For whole blood these figures should be somewhat smaller (about five-sixths). For the purposes of our experiments analyses of plasma would however be much less significant than whole blood.

In the production and maintenance of the low level as it exists probably in acidosis of renal or metabolic origin the alkali of the

[4] Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 294.

Digitized by Google

blood by influencing the respiratory center controls the CO_2 (H_2CO_3). On the other hand in the low and high levels produced by disturbances of breathing the respiratory center by controlling the CO_2 exerts a powerful influence upon the alkali ($NaHCO_3$).

If objection be taken to our saying that C_H may be "normal" at various levels of CO_2, we would point out that people living 5,000 or 10,000, or even 15,000 feet above sea level, or again below sea level, have as much right to consider themselves normal as have we who live by the sea. But their levels of CO_2 are widely different from ours.[5]

Method.

All experiments were performed on healthy dogs. The femoral artery was exposed under local anesthesia (cocaine 0.5 cc. of 2 per cent solution) and a cannula inserted. Samples of blood, 3 to 6 cc. each, were withdrawn at regular intervals with proper precautions to avoid getting stagnant blood. Clotting was prevented by a small amount of powdered potassium oxalate. 1 cc. of the whole blood was analyzed to determine its CO_2 content. The remainder of the sample was then brought into equilibrium at 20°C. with air containing 5.4 to 5.7 per cent CO_2 (determined by analysis). 1 cc. of this whole blood was then analyzed. This gave the CO_2 capacity. The methods were those described by Henderson and Morriss.[6] The figures for CO_2 capacity are proportional and closely approximating but not absolutely identical with those which would be computed by the Van Slyke method. We prefer the expression "CO_2 capacity," to the still rather hypothetical expression "alkaline reserve." The capacity of the proteins to combine with CO_2 may be considerable (Buckmaster[7]). By CO_2 capacity we mean the total amount of CO_2 (reduced to 0° and 760 mm.) in the blood whether in solution or combined with alkali or protein at the tension of the CO_2 in the air with which the sample of blood is brought into equilibrium.

The alveolar air was determined with a mask and bag by the Higgins-Plesch method.

[5] Fitzgerald, M. P., *Phil. Tr. Roy. Soc., Series B*, 1913, cciii, 351; *Proc. Roy. Soc., Series B*, 1914–15, lxxxviii, 248.

[6] Henderson and Morriss, *J. Biol. Chem.*, 1917, xxxi, 217.

[7] Buckmaster, G. A., *J. Physiol.*, 1917, li, 164.

Digitized by Google

The reliability of all our work depends upon the accuracy with which the blood was "saturated" or (better) "equilibrated" with air containing 5.5 per cent CO_2. The method of equilibration and analysis was therefore subjected to the following tests.

A supply of arterial blood was drawn. Analysis was made for (*a*) the CO_2 content. A sample was then equilibrated with air containing 5.5 per cent CO_2 and analyzed (*b*). A second portion of the blood was then equilibrated with air containing 10 per cent CO_2. Part was analyzed (*c*), and the rest was then equilibrated with 5.5 per cent CO_2 and analyzed (*d*). A third sample was equilibrated with atmospheric air. A part of this was then analyzed (*e*), and the remainder was equilibrated with 5.5 per cent CO_2 and analyzed (*f*). In all cases the equilibration was carried out by the method used in routine determinations of the CO_2 capacity. The equilibrating air was passed through a tube into a test-tube containing 2 or 3 cc. of blood under albolene until the liquid was violently agitated for 30 seconds and for the most part in foam. The test-tube was then stoppered and set aside until the blood and oil separated.

The foregoing series of equilibrations and analyses was repeated with blood drawn from the same dog several hours later. The equilibrations lasted 2 minutes each. The results obtained were as follows:

	a	*b*	*c*	*d*	*e*	*f*
First series.....	47	50	60	51	37	50
Second "	47	47	58	47	37	49

Under the extreme conditions the concordances 50, 51, 50 in one series and 47, 47, 49 in the other (*b*, *d*, and *f*) are satisfactory.

Object of the Experiments.

Our aim was to determine the effect upon the CO_2 capacity of the blood induced by increasing the CO_2 tension of the pulmonary air. This was effected by depressing respiration by means of morphine or by administering CO_2 in proper dilution in the air breathed. For the latter purpose a Douglas bag of 50 liters capacity was filled with air to which 5 or 6 per cent of CO_2 was added. The animal rebreathed this mixture through a

Digitized by Google

closely fitting mask and as the CO_2 increased oxygen was added in equal amount.

EXPERIMENTAL.

Two control experiments were performed. They demonstrate that under the conditions all the functions remain nearly constant. Even in the slight variations observed the CO_2 content of the blood shows a tendency to follow the alveolar CO_2 within 5 minutes. With a distinct lag of 15 to 30 minutes the CO_2 capacity follows the CO_2 content.

Experiment 1.—Control. Dog, male, 12 kilos. No drug. Animal quiet and comfortable, and breathing normally throughout. Air used for equilibrating blood contained 5.4 per cent CO_2.

Time.	Conditions.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
4.15	Normal.	5.4	46	47	39.8
4.45	"	5.5	48	47	39.8
5.15	"	5.2	44	45	39.8
5.45	"	5.4	50	47	39.7
6.15	"	5.2	50	50	39.8
6.45	"	5.3	48	46	39.8
7.15	"	5.4	49	50	39.6

Experiment 2.—Control. Dog, male, 9 kilos. Conditions as in Experiment 1. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	*°C.*
10.15 a.m.	Normal.	5.3	47	49	39.7
10.45 "	"	5.2	47	48	39.7
11.15 "	"	5.4	50	48	
11.45 "	"	5.1	48	48	39.7
12.15 "	"	5.5	51	50	
12.45 "	"	5.5	51	49	39.8
1.45 p.m.	"	5.4	51	52	
2.15 "	"	5.4	50	50	39.7

Digitized by Google

In the next four experiments, after a preliminary normal period in each case, morphine was administered subcutaneously. The extent of the depression of respiration may be judged by the rise in the alveolar CO_2 from the normal of about 5.3 up to amounts ranging from 6.1 to 6.9. In one case (Experiment 6) this rise was comparatively slight at first (owing to the animal being of an excitable character and breathing correspondingly) and in the columns showing the CO_2 content and CO_2 capacity the effects are correspondingly slight. Later in this experiment and in all of the experiments in which the alveolar CO_2 attained a high level, the CO_2 content of the blood invariably followed it, and the CO_2 capacity followed the CO_2 content with a lag of 20 to 30 minutes. Thus the CO_2 content rose from about 50 up to values ranging from 63 to 67, and the CO_2 capacity from about 50 up to values of 59 to 64.

Experiment 3.—Dog, male, 14 kilos. Morphine administration, depressed respiration, followed by rise of CO_2 content and CO_2 capacity. Equilibrating air 5.7 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
9.30 a.m.	Normal.	5.3	52	52	39.9
10.00 "	"		52	53	
10.05 "	Morphine, 0.14 gm.				
10.15 "	Vomiting and panting.				
10.40 "	Quiet.	6.6	57	48	
11.20 "			60	53	37.8
12.00 "	Quiet.	6.6	62	54	
12.30 "	"		62	56	
1.30 p.m.	"	6.9	67	59	
2.10 "	"		63	60	36.6
2.40 "	"		58	59	
3.30 "	"	6.8	64	62	
6.00 "	Struggling.	4.8	49	56	37.0

Digitized by Google

Experiment 4.—Dog, male, 9 kilos. Morphine administration. Conditions as in Experiment 3. Equilibrating air 5.7 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
11.30 a.m.	Normal.	5.4	49	50	39.7
11.35 "	Morphine, 0.16 gm.				
12.15 "		5.9	57	54	
12.40 "		6.0	60	56	38.8
1.20 p.m.		6.3	62	59	
2.00 "		6.7	64	59	
2.40 "		6.6	63	60	38.6

Experiment 5.—Dog, male, 12 kilos. Morphine administration. Conditions as in Experiment 3. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
10.15 a.m.	Normal.	5.3	50	51	39.6
10.20 "	Morphine, 0.24 gm.				
10.50 "		6.0	56	54	
11.35 "		6.6	64	58	38.4
12.30 "			63	60	
1.30 p.m.		6.7	65	59	
3.00 "	Very quiet.		70	64	

Experiment 6.—Dog, male, 10 kilos. Morphine administration. Conditions as in Experiment 3. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Alveolar CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
10.00 a.m.	Normal.	5.4	49	49	39.8
10.30 "	Morphine, 0.16 gm.				
11.15 "			54	48	
12.00 "		5.8	56	52	39.0
1.15 p.m.			60	56	
2.00 "		6.1	63	58	
3.00 "			64	58	
4.00 "			62	59	38.8

Digitized by Google

In Experiment 7 morphine was administered and the animal was then made to rebreathe (by means of a mask and Douglas bag) air of a CO_2 content at first practically the same as the normal alveolar air and rising gradually to 8.2 per cent. The CO_2 content of the blood rose in 1 hour and 10 minutes to 76 and the CO_2 capacity to 67.

Experiment 7.—Dog, male, 8 kilos. Morphine and CO_2 administration. Procedure same as Experiments 3 to 6 except that dog rebreathed into a large bag, which in the beginning contained 5 per cent CO_2. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
3.35	Normal.		48	50	39.9
4.00	Morphine, 0.12 gm.				
4.20	Breathing CO_2.	5.1			
4.40	" "		70	56	
5.10	" "		69	64	40.0
5.15	" "	8.2			
5.30	" "		76	67	
6.00	" "		74	66	

Finally three experiments were performed in which no morphine was administered, but the rebreathing from the bag was begun with about 6 per cent of CO_2 and continued until the CO_2 had accumulated up to 10, 11, and 20 per cent respectively. (Ample oxygen was supplied.) Of course the breathing was stimulated to violent activity at percentages between 6 and 10, but much less above 10 per cent. The effects on the blood were of exactly the same character as in the experiments in which respiration was depressed and the alveolar CO_2 raised by means of morphine. The CO_2 content was forced up (to 74, 73, and 87) and the CO_2 capacity followed it upward (to 64, 59, and 74). When the rebreathing was stopped and the animal again allowed to breathe normal air, the CO_2 content led the CO_2 capacity downward toward normal values again, but the descent was slow and gradual.

Digitized by Google

Experiment 8.—Dog, male, 8 kilos. CO_2 administration. Procedure same as Experiment 7, except that morphine was omitted. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
4.15	Normal.		51	50	39.8
4.30	CO_2 started.	6.0			
4.45			54	55	
5.15		10.2	68	59	39.9
5.45		11.4	72	64	
6.15			74	63	
6.20	CO_2 stopped.				
6.45			64	67	

Experiment 9.—Dog, female, 14 kilos. CO_2 administration. Procedure similar to Experiment 8. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
p.m.		*per cent*	*vol. per cent*	*vol. per cent*	*°C.*
1.40	Normal.		48	46	39.8
2.15	CO_2 started.	6.0			
2.45		8.2	62	54	39.7
3.15			66	56	39.6
3.45		10.3	73	59	
4.15			72	61	39.8
4.20	CO_2 stopped.				
4.45			54	59	
5.15			50	54	
5.45			47	52	

Digitized by Google

Experiment 10.—Dog, male, 11 kilos. CO_2 administration. Procedure same as Experiments 8 and 9, but the CO_2 in bag was run up higher. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Bag of air CO_2.	Blood CO_2.		Rectal temperature.
			Content.	Capacity.	
		per cent	*vol. per cent*	*vol. per cent*	°C.
11.30 a.m.	Normal.		48	50	39.9
11.45 "	CO_2 started.	6.2			
12.15 "			57	54	
1.15 p.m.			67	60	
2.15 "		10.3	70	62	40.1
2.45 "			72	66	
3.15 "		14.0	79	65	
4.00 "			87	71	
4.45 "		20.0	86	74	42.0
4.50 "	CO_2 stopped.*				

* After drawing a few breaths of fresh air the dog stopped breathing. The heart continued to beat for several minutes and then stopped. Died.

Experiment 10 is extraordinary. The CO_2 in the air breathed reached 20 per cent. The CO_2 content of the blood rose to 86 and the CO_2 capacity to 74. When the animal was finally allowed to breathe normal air again its respiration ceased after a few breaths and a fatal apnea followed. Apparently the alkali content of the blood had been raised so high that when the abnormally high pressure of CO_2 was withdrawn the respiratory center lacked an adequate stimulus.

CONCLUSIONS.

Whenever by means of morphine respiration is depressed and the alveolar CO_2 is raised, or whenever by the administration of CO_2 in the air breathed the CO_2 content of the blood is forced up, the CO_2 capacity (alkaline reserve) of the blood also rises. This rise is probably due to a compensatory passage of alkali from the tissues into the blood. Through this mechanism respiration exerts a powerful influence upon the "alkaline reserve" of the blood.

Digitized by Google

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

II. LOW LEVELS OF CO_2 AND ALKALI INDUCED BY ETHER. THEIR PREVENTION AND REVERSAL.

By YANDELL HENDERSON and H. W. HAGGARD.

(From the Physiological Laboratory, Yale Medical School, New Haven.)

(Received for publication, December 31, 1917.)

In the light of present knowledge it appears probable that many, perhaps most, of the functional disturbances induced by anesthesia are due to the production of an abnormal CO_2 capacity (alkaline reserve) in the blood, or to deeper effects of which the CO_2 capacity is an index.

At present such effects are generally interpreted as "acidosis." Our observations show that, at least under ether, they are of a very different origin.

Morriss[1] has shown that in patients under ether a lowering of the alkaline reserve of the plasma occurs. On the basis of some preliminary experiments on dogs Prince and the authors[2] have verified Morriss' observation and have obtained results indicating that the effect is due to the respiratory excitement induced by ether. An abnormally great amount of CO_2 is ventilated out of the blood by the excessive breathing and the CO_2 content is thus lowered. Sometimes when this blowing off has been very rapid the result is failure of respiration: apnea vera. When the blowing off is less rapid but is prolonged another change occurs which (as Prince first pointed out to us) is of a compensatory character. This consists (as set forth in general terms in the first paper of this series) in a decrease of the CO_2-combining power (alkaline reserve) of the blood. The simplest supposition upon which to explain it is that when the alkalinity

[1] Morriss, W. H., *J. Am. Med. Assn.*, 1917, lxviii, 1391.

[2] Henderson, Y., Prince, A. L., and Haggard, H. W., *J. Am. Med. Assn.*, 1917, lxix, 965.

Digitized by Google

of the blood is abnormally increased by the blowing off of CO_2, alkali passes out of the blood into the tissues. As a result of these two processes both the CO_2 and the alkali in the blood are decreased, and the proportion of H_2CO_3:$NaHCO_3$ and consequently the C_H of the blood are much less decreased than would be the case if only the CO_2 were altered.

If the "level of CO_2 and alkali" (as defined in the previous paper) is reduced below the critical value lying between 33 and 36 volumes per cent of CO_2, a condition of general depression of all vital functions results. Above the critical level the process appears to be reversible, for if CO_2 is administered in the air breathed and the CO_2 content of the blood is thus raised the CO_2 capacity follows it upward, probably because of the passage of the alkali from the tissues back into the blood. On the other hand if the marked resistance to further depletion of CO_2 capacity which occurs at the critical level is broken down and the CO_2 capacity further reduced, the result is a condition of vital depression from which the subject does not spontaneously recover. This condition may be termed acapnial shock.

In the experiments to be reported here the methods were identical with those in the first paper of this series, and the controls reported there apply equally here.

EXPERIMENTAL.

In the first experiment are shown the effects (in the dog) of careful and skilful etherization by the open method. The initiation of anesthesia was rapid, the administration as nearly uniform as practicable by this method, and both excitement of respiration from too little ether and depression from too much were avoided. The CO_2 content was reduced in the course of the 1st hour from the normal of 50 down to 45. It underwent no considerable change during the succeeding 5 hours.

Digitized by Google

Experiment 1.—Dog, male, 11 kilos. Skilful etherization. Full anesthesia by open method. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
			vol. per cent	*vol. per cent*
8.30 a.m.	Normal.	21	51	50
9.00 "	Ether.			
9.30 "		47	43	50
10.00 "		48	43	45
10.30 "		26	46	46
11.00 "		30	51	48
11.30 "		17	47	47
12.00 "		19	44	47
12.30 "	Shallow respirations.	29	56	48
1.00 p.m.	Less ether.	21	46	45
1.30 "		14	48	44
2.00 "		19	50	44
2.30 "		29	44	46
3.00 "		25	41	45
3.05 "	Experiment stopped.	Dog in good condition.		

In the second experiment the initiation of anesthesia was purposely made unskilfully slow. The dog was allowed to struggle in the stage of excitement for a considerable time, and when this period came to an end the ether was given irregularly so that at no time was there deep anesthesia.

Experiment 2.—Dog, female, 8 kilos. Etherization, open method, excessive respiration. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p.m.			*vol. per cent*	*vol. per cent*
4.00		18	47	49
4.24	Ether started.			
4.26		44		
4.28		68		
4.30		74		
4.33		90		
4.35			38	48
5.00		62		
5.10			39	44
5.30		34		
6.10			40	43
6.30		46		
7.10			38	41

Digitized by Google

In the third experiment is afforded an example of extremely bad light etherization, yet which was not so bad as to cause early death from failure of breathing (apnea). The excessive breathing reduced the CO_2 content of the blood progressively and the CO_2 capacity followed downward with the usual lag until at a CO_2 capacity of 28 the animal was moribund. It died soon after.

Experiment 3.—Dog, male, 14 kilos. Prolonged ether excitement. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
			vol. per cent	*vol. per cent*
9.20 a.m.	Normal.	19	54	51
9.30 "	Ether started.			
10.00 "		63	44	52
10.30 "		82	39	44
11.00 "		70	36	43
11.30 "		78	37	44
12.00 "		42	36	41
12.30 "		84	35	39
1.00 p.m.		59	37	38
1.30 "		76	31	36
2.00 "		56	22	37
2.30 "		59	24	31
3.00 "		82	27	28
3.15 "	Moribund. Ether stopped.			
3.19 "	Stopped breathing.			
3.24 "	Heart stopped. Dog dead.			

In the fourth experiment the same procedure was followed until the CO_2 content had fallen at the end of 210 minutes to 35 volumes per cent and the CO_2 capacity to 38. The administration of ether was then stopped, and in the course of the succeeding 90 minutes the CO_2 content rose again nearly to normal and thereby caused the CO_2 capacity to rise to 46, only a little below the normal level.

Digitized by Google

Experiment 4.—Dog, male, 10 kilos. Light and irregular ether for 210 minutes, with spontaneous recovery toward normal during following 90 minutes. Equilibrating air 5.7 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
			vol. per cent	*vol. per cent*
9.30 a.m.		20	50	53
9.45 "	Ether started.			
10.00 "		82		
10.15 "			42	50
10.30 "		68		
10.45 "			36	47
11.00 "		66		
11.15 "			37	44
11.30 "		69		
11.45 "			36	41
12.00 "		59		
12.15 "			40	38
12.30 "		60		
1.15 p.m.			35	38
1.20 "	Ether stopped.			
1.45 "			43	39
2.15 "			49	43
2.45 "			47	46
2.50 "	Animal in good condition.			

Evidently in the foregoing experiment the decrease of the CO_2 capacity (alkaline reserve) was spontaneously reversible from the low level reached.

The first part of Experiment 5 was similar to the preceding experiment. After the CO_2 capacity had been reduced to 39 in this case however, instead of discontinuing the ether the amount administered was increased to a maximum just short of causing respiratory failure. This condition was continued for 80 minutes. Under the depression of respiration thus caused the alveolar CO_2 was of course increased. The CO_2 content of the blood rose to 67 and the CO_2 capacity was raised to 54; that is, slightly above normal. The animal died soon after, probably from an insufficiency of oxygen.

Digitized by Google

Experiment 5.—Dog, male, 12 kilos. Light and irregular etherization followed by very deep etherization (with no rebreathing). Equilibrating air 5.65 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p. m.			*vol. per cent*	*vol. per cent*
3.00		21	49	50
3.15	Ether started.			
3.45		82	39	47
4.15		73	37	42
4.45		57	38	43
5.15		61	36	40
5.45		63	39	39
6.00	Ether increased to limit.			
6.15		47	43	39
6.45	Blood dark.	37. shallow.	53	41
7.15	" "	46 "	67	54
7.23	Dog dead.			

From this experiment it is clear that when the CO_2 content is lowered by etherization the effect is not due to any influence of the ether *per se* other than the excessive pulmonary ventilation and low alveolar CO_2. In order however to make absolutely sure of this point we performed Experiments 6 and 7. We reasoned that if ether acts in some obscure way upon metabolism to induce "acidosis" (the opinion now widely held), then the more ether administered the greater should be the so called acidosis and the lower the CO_2-combining power. As ether in low concentrations excites and in high concentrations depresses respiration, it was easy to frame a crucial experiment. Thus in Experiments 6 and 7 an etherization so profound was maintained that respiration was depressed and the CO_2 content rose instead of falling. As the protocols show the CO_2 capacity followed upward, rising from 50 to 61 in one case, and from 47 to 60 in the other.

Digitized by Google

Experiment 6.—Dog, male, 9 kilos. Profound etherization and depressed respiration. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p. m.			*vol. per cent*	*vol. per cent*
3.30	Normal.	16	52	50
3.45	Ether started, pushed rapidly.			
4.15		11	60	54
4.30	Shallow respirations. Blood dark.	16	57	56
4.45	" " " "	22	57	55
5.15	" " " "	8	60	61
5.20	Breathing stopped. Dog dead.			

Experiment 7.—Dog, male, 7 kilos. Similar to Experiment 6. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.	
			Content.	Capacity.
p. m.			*vol. per cent*	*vol. per cent*
6.10	Normal.	21	47	47
6.30	Ether started. Administered rapidly.			
7.00	Respirations very shallow.	72, blood dark.	64	63
7.05	Ether lightened.			
7.30	Shallow respirations.	32 " "	59	60
7.45	" "	40 " "	63	60
7.50	Dog in critical condition. Ether stopped.			
7.52	Three or four more breaths, then breathing stopped, heart beating.			
7.56	Heart stopped. Dog dead.			

Even Experiments 6 and 7, decisive as they appear in contrast to those preceding them, *e.g.*, Nos. 2, 3, 4, and 5, might leave open the possibility that the excitement of light ether causes a depression of the CO_2 capacity in some way (*e.g.*, excessive adrenal secretion) other than by the blowing off of CO_2. Accordingly we performed Experiments 8 and 9 in which light etherization was maintained for several hours but loss of CO_2 was prevented by the fact that the air which the animal breathed was enriched by the addition of CO_2 in the amounts shown in the protocols. It will be seen that in both cases the CO_2 capacity

Digitized by Google

was slightly elevated and that when the experiments were discontinued, after $5\frac{1}{2}$ and $4\frac{1}{4}$ hours respectively, the animals were in excellent condition.

Experiment 8.—Dog, male, 9 kilos. Light etherization with CO_2 inhalation. Tracheal cannula connected with closed circuit ether bottle. Air containing approximately 7 per cent of CO_2 administered through ether. Dog at all times was kept under very light ether as determined by periods of struggling, vigorous breathing, and full reflexes. Equilibrating air 5.45 per cent CO_2.

Time.	Condition.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p. m.		*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
3.30	Normal.	0	49	51	
4.00	Under ether.	0	42	49	170
4.05	CO_2 started.	7.5			
4.15		7.5	58	50	168
4.45		7.1			
5.00		3.8	52	55	150
5.30		7.7	63	57	150
6.15		7.6	55	55	154
6.45		7.0	60	57	160
7.15		6.8	56	56	150
7.45		6.8	53	54	160
	Dog in excellent condition.				

Experiment 9.—Dog, male, 14 kilos, young. Similar to Experiment 8. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p. m.		*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
2.48	Normal.	0	48	47	
3.15	Ether started.				
3.42		0			128
3.45	CO_2 started.	6.2			
4.45		6.2	63	56	116
5.45		6.0	68	54	110
6.45		7.7	71	51	114
7.45		6.4	62	56	110
8.45		7.1	67	55	108
	Dog in good condition. Blood a good bright normal color.				

Digitized by Google

CONCLUSIONS.

The disturbance of the CO_2 capacity of the blood by ether appears from these experiments to be wholly dependent on disturbance of respiration. If the anesthesia is managed so that respiration is but little increased, the lowering of the CO_2 capacity of the blood is slight. Ether hyperpnea however causes a very great reduction. Down to the critical level between 33 and 36 volumes per cent the process is spontaneously reversible and the animal recovers. Below this level it appears to be irreversible, and death ensues.

At present such effects even above the critical level are generally interpreted as "acidosis." Our results show them to be of a very different origin. It is possible however that below the critical level a true acidosis of an asphyxial character sets in.

Etherization so profound as to depress respiration causes a rise of the CO_2 capacity.

Light etherization such as is otherwise most effective in lowering the CO_2 capacity, and most harmful to the subject, loses this influence when administered with sufficient CO_2 to maintain the alveolar CO_2 at a normal level.

From these facts it is clear that under ether the CO_2 capacity of the blood (alkaline reserve) follows and is controlled by the CO_2 content, and that the CO_2 content is in turn dependent upon the alveolar CO_2, which is determined by the breathing.

These results afford, we believe, final proof of the essential correctness of the views on this topic (acapnia under anesthesia[3]) which have been advocated now for 10 years past in papers from this laboratory.

We desire to acknowledge our debt to Van Slyke and his collaborators[4] for bringing forward the conception of the "alkaline reserve," or as we prefer to call it the CO_2 capacity of the blood. This conception has enabled us to produce proof on a topic which after controversy and general rejection was evidently passing into oblivion.

[3] Bryant, J., and Henderson, Y., *J. Am. Med. Assn.*, 1915, lxv, 1 (discussion of clinical aspects of problem and references to previous work).

[4] Van Slyke, D. D., Stillman, E., and Cullen, G. E., *J. Biol. Chem.*, 1917, xxx, 401.

Digitized by Google

Digitized by Google

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

III. THE EFFECTS OF EXCESSIVE PULMONARY VENTILATION.

By YANDELL HENDERSON and H. W. HAGGARD.

(*From the Physiological Laboratory, Yale Medical School, New Haven.*)

(Received for publication, December 31, 1917.)

Pain causes excessive breathing: abnormally intense pulmonary ventilation. What part does this ventilation play as an intermediary between pain and its end results; *e.g.*, the characteristic alterations of the CO_2 capacity of the blood, of venous and arterial pressure, of oxygen consumption, etc.? For an answer to this question we need experiments which afford excessive pulmonary ventilation but none of the other conditions of pain. In man this test may be made experimentally (so far as one dares and is physically able) by prolonged voluntary forced breathing.[1] In animals the problem may be attacked by means of excessive artificial respiration. In this paper we shall report some experiments on the latter line of attack.

There seems to be a misapprehension as to what we mean by excessive breathing. One observer writes us from a casualty clearing station in France as follows:

"As to excessive breathing, we have made careful observations on freshly wounded men both in the trenches and in the casualty clearing station, and have found that *it does not occur*. A man was brought in a few minutes after he had been hit by a small splinter of a bomb which had penetrated his wrist. He was suffering intense pain and was breathing, each expiration a grunt, twelve times a minute."

We have tried breathing in this way ourselves and find that after doing so for 40 seconds an apnea of 20 seconds followed. Quantitatively this indicates that, at least as we did it, such breathing involves a 50 per cent overventilation. The same writer says, "The severely wounded commonly testify to feeling no pain." This, we think, corresponds with the

[1] Henderson, Y., *Am. J. Physiol.*, 1909–10, xxv, 321; *Bull. Johns Hopkins Hosp.*, 1910, xxi, 236; *J. Pharm. and Exp. Therap.*, in press.

Digitized by Google

fact that, as reported, shock occurs in only a minority of the wounded. Furthermore overventilation quickly results in a numbing of sensation,—an almost anesthetic condition as regards consciousness, and yet the excessive breathing may continue.

Method.

In general the methods employed were similar to those in the preceding papers of this series. The animals were however given an intramuscular injection of chloratone dissolved in alcohol. At the outset of each experiment they were also etherized for a few minutes.

The apparatus with which the excessive artificial respiration was administered is shown in Fig. 1. It consisted of two rotary air blowers belted to a shafting (not shown) so that one forced air toward a Y tube tied into the trachea and the other sucked air from it. Escape valves were connected with each side to prevent the pressures either positive or negative ever exceeding 30 cm. water column. By means of a hand lever the Y tube in the trachea was opened alternately to pressure and suction.

EXPERIMENTAL.

In a control experiment without artificial respiration it was found that chloratone itself in the dosage employed has no marked effect upon either the CO_2 content or CO_2 capacity of the blood. It is well known that dogs under chloratone have a low arterial pressure.

Experiment 1.—Control. Dog, male, 19 kilos. Chloratone anesthesia. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Blood CO_2.	
		Content.	Capacity.
		vol. per cent	*vol. per cent*
10.30 a.m.	Normal.	48	49
10.45 "	Chloratone administered, 4.8 gm.		
11.35 "		46	48
1.35 p.m.		47	46
2.35 "		48	46

Digitized by Google

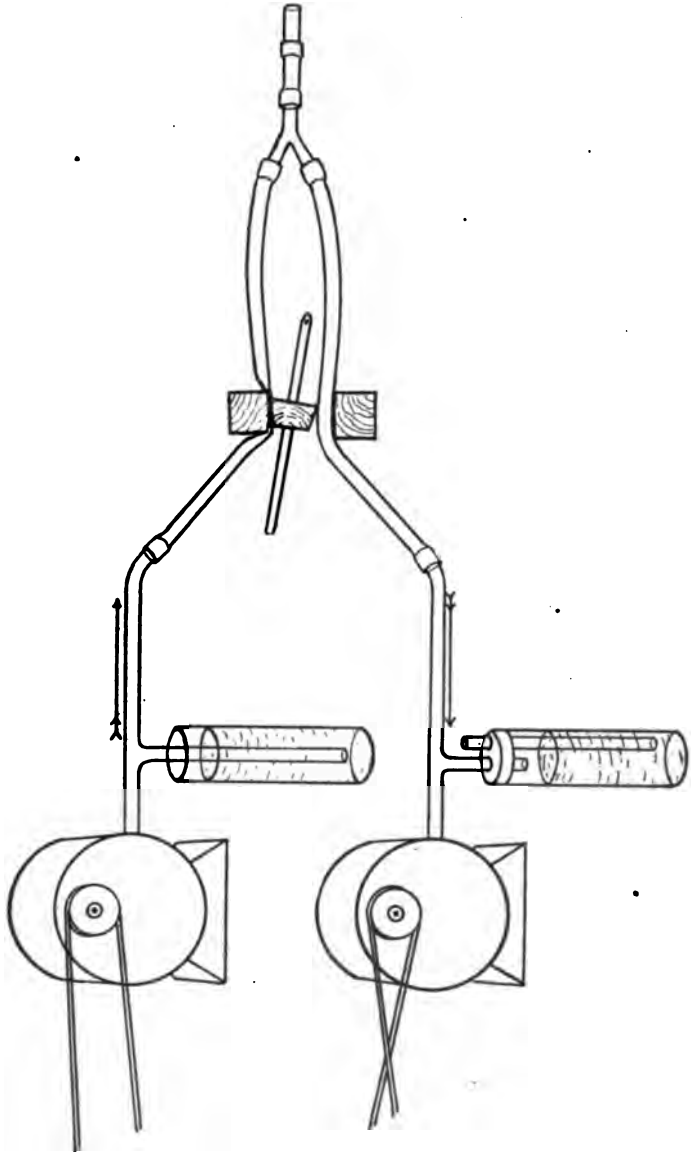


Fig. 1.

Digitized by Google

In Experiment 2 the artificial respiration was made as vigorous as possible. The instant the lungs were filled or emptied the lever was reversed and the alternate phase of respiration induced. From the protocol it appears that within 22 minutes the CO_2 content of the blood was reduced from 52 to 22, the CO_2 capacity from 50 to 38, and the arterial pressure from 130 mm. down to 50. When the ventilation was stopped it was necessary to administer artificial respiration by hand by squeezing the chest occasionally in order to prevent death from prolonged apnea. Even so, all of the functions measured continued to fall, the CO_2 capacity to 28 and arterial pressure to 25, and the animal died 55 minutes after the artificial respiration was stopped. In all of these experiments a careful autopsy was conducted, air embolism and pulmonary hemorrhages being particularly looked for. The findings in this respect were negative. In this animal the only abnormality found was a mass of roundworms in the right heart.

Experiment 8.—Dog, male, 8 kilos. Passive overventilation. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
		vol. per cent	vol. per cent	mm.
10.15 a.m.	Normal.	53	51	
10.30 "	Chloratone, 2 gm.			
11.35 "	Operated.	52	50	130
11.43 "	Ventilation started.			
11.50 "				110
11.55 "				50
12.05 "	Ventilation stopped.			
12.06 "		22	38	
12.20 "	Reflexes hyperactive.			25
12.30 "		20	28	25
12.35 "	Reflexes practically absent. Respirations 36 per min.			
1.00 p.m.	Dead.			

In Experiment 3 the artificial respiration was carried on much less vigorously than in the preceding case, but was still markedly in excess of normal breathing. In the course of an hour and

Digitized by Google

45 minutes the CO_2 content of the blood was reduced to 28 volumes per cent, the CO_2 capacity to 34, and the arterial pressure to 70 mm. After termination of the mechanical artificial respiration it was found necessary for a time to squeeze the chest occasionally to prevent death in apnea.

In the course of half an hour spontaneous respiration of a shallow rapid type returned, but the animal continued to sink and died an hour and 40 minutes after the cessation of the period of excessive ventilation.

In this and in the preceding experiment it was observed that at first the reflexes, particularly the knee-jerk, seemed to be actually increased in responsiveness, but that after the excessive artificial respiration was stopped the reflexes decreased in responsiveness until they had practically disappeared.

Experiment 3.—Dog, male, 7 kilos. Passive overventilation, similar to Experiment 2, but not so active. Equilibrating air 5.6 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
p. m.		*vol. per cent*	*vol. per cent*	*mm.*
3.00	Normal.	55	56	
3.20	Chloratone, 1.4 gm.			
4.50		54	55	130
5.15	Ventilation started.			
5.30		37	41	130
6.00		31	37	120
6.15	Reflexes hyperactive.			
6.30		31	35	95
7.00		28	34	70
7.05	Ventilation stopped. Artificial respirations 3 min. (manual).			
7.30	Reflexes partially abolished.			
7.30		23	38	50
8.00	Respirations shallow and rapid, 44 per minute.			
8.00				50
8.30	Respirations, 55 per min.			50
8.35		26	35	48
8.47	Dog dead.			

Autopsy.—No pulmonary lesions. No air emboli in coronary vessels.

Digitized by Google

Experiment 4 affords evidence that excessive ventilation may have profound effects, including circulatory failure, which may continue even when the CO_2 capacity returns to normal,—unless perhaps this animal was unusually susceptible to chloratone. Death was evidently due to circulatory failure.

Experiment 4.—Dog, male, 9 kilos. Overventilation. Similar to Experiment 3. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
		vol. per cent	*vol. per cent*	*mm.*
9.00 a.m.	Normal.	47	50	
9.20 "	Chloratone, 1.8 gm.			
10.20 "		49	48	100
10.35 "	Ventilation started.			
10.50 "		29	42	70
11.20 "		21	37	70
11.50 "		34	40	60
12.20 "	Reflexes hyperactive.			
12.20 "		33	36	60
1.45 p.m.	Ventilation stopped.			
1.45 "		30	38	60
2.15 "		43	44	58
2.30 "	Oxygen lack. Blood dark. Occasional gasps.			
3.15 "	Reflexes sluggish.	43	44	50
3.40 "	Irregular blood pressure.			50-90
4.00 "	Great O_2 deficiency.	60	54	35
4.10 "	Dog dead.			

Autopsy.—Nothing abnormal found.

From the two preceding experiments it is clear that excessive artificial respiration results in a marked decrease in the CO_2 content and CO_2 capacity of the blood and also in the arterial pressure. In the light of the experiments reported in the two preceding papers of this series it is altogether likely that these results are chiefly due to the excessive ventilation. A possible objection may however be made that the violent movements of excessive artificial respiration are in part responsible for the effects,

Digitized by Google

particularly upon arterial pressure. In order to test this point therefore we carried out Experiment 5. The point of the experiment was to administer quite as vigorous artificial respiration as in the previous experiments, but with air containing such a percentage of CO_2 as would prevent any reduction in the CO_2 content of the blood.

For this purpose the apparatus shown in Fig. 1 was used with

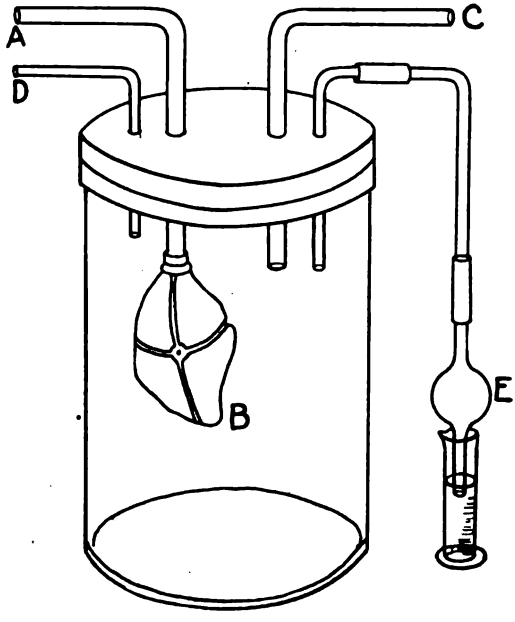


FIG. 2.

the addition of that shown in Fig. 2. The latter consisted of a glass jar of 12 liters capacity in which a rubber bag (B) was suspended on the end of a tube (A) which was fastened to the Y tube of the apparatus of Fig. 1. Tube C was attached to the animal's trachea. Oxygen was passed into the jar as needed, and gas samples were taken, through tube D, while a small escape valve (E, at the right of the figure) allowed a little fresh air to enter and leave the jar at each reversal of the air current.

Digitized by Google

Experiment 5.—Control experiment. Dog, male, 9 kilos. Excessive artificial respiration with air containing CO_2. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Blood CO_2.		Arterial pressure.
		Content.	Capacity.	
p. m.		*vol. per cent*	*vol. per cent*	*mm.*
2.10	Normal.	47	49	
2.30	Chloratone, 1.8 gm.			
3.00	Ether. Operated.			
3.21	" stopped.	43	46	
3.40				110
3.45	Ventilation started.			
3.50				138
4.15		56	52	
4.40	Contents of rebreathing chamber 7.9 per cent CO_2.			
4.45		58	53	
4.45				142
5.10	Contents of rebreathing chamber 6.7 per cent CO_2.			
5.15		56	54	126
5.22	Ventilation stopped.			
5.45	Respirations, 32 per min.			118
5.55		38	44	90
6.15	Respirations, 37 per min.			75
6.30	" 32 " "			80

Reflexes normal, animal in good condition. Killed.

From this experiment it appears that the movements of excessive artificial respiration so far from causing a fall of arterial pressure in themselves tend to cause (or at least are compatible with) a rise, due either to the pumping action upon the thoracic and abdominal blood vessels or to the effects of high CO_2. The rather low arterial pressure after termination of the period of excessive ventilation may fairly be assigned to the chloratone.

CONCLUSIONS.

Excessive pulmonary ventilation by means of artificial respiration induces not only a lowering of the CO_2 content but also of the CO_2 capacity of the blood. Arterial pressure also falls and death

Digitized by Google

from circulatory failure may follow[2]. If the artificial respiration, instead of being made with fresh air, is carried on by continual reinjection mainly of expired air, so that the CO_2 content of the blood is not reduced, the CO_2 capacity and arterial pressure do not fall and the other ill effects also fail to appear.

[2] Ewald, A., *Arch. ges. Physiol.*, 1873, vii, 580. Henderson, *Am. J. Physiol.*, 1908, xxi, 126.

Digitized by Google

Digitized by Google

RESPIRATORY REGULATION OF THE CO_2 CAPACITY OF THE BLOOD.

IV. THE SEQUENCE OF TRAUMA, EXCESSIVE BREATHING, REDUCED CO_2 CAPACITY, AND SHOCK.

By YANDELL HENDERSON and H. W. HAGGARD.

(*From the Physiological Laboratory, Yale Medical School, New Haven.*)

(Received for publication, December 31, 1917.)

The term "shock" has come to be applied not only to the most diverse sorts of functional depression following trauma, but also to nearly any experimental method (no matter how artificial) for causing circulatory failure.

The common procedure for the production of shock experimentally in dogs is to expose and manipulate the abdominal viscera under morphine anesthesia. The bearing of such radical experiments on shock in man can be accepted only with considerable reserve. This is important in considering experimental work on shock because of the fact that, as Mann[1] has pointed out, with the exception of certain experiments perform ed some years ago in this laboratory, the procedure of exposure and manipulation of the abdominal viscera has been almost the sole method by which "shock" has been induced experimentally.

In some experiments, of which a preliminary notice was published some months ago by Prince and ourselves,[2] it was found that after irritation of the abdominal viscera a marked reduction of the CO_2 capacity of the blood occurred. In these experiments we intentionally induced excessive (natural) breathing.

Recently Cannon[3] has reported that in wounded soldiers in shock the "alkaline reserve" or CO_2 capacity of the blood is

[1] Mann, F. C., *Bull. Johns Hopkins Hosp.*, 1914, xxv, 205.

[2] Henderson, Y., Prince, A. L., and Haggard, H. W., *J. Am. Med. Assn.*, 1917, lxix, 965.

[3] Cannon, W. B., Reports to Committee on Physiology, National Research Council, *Am. J. Physiol.*, in press.

Digitized by Google

much reduced, that a further reduction is induced by anesthesia and operation, and that administration of alkali intravenously is beneficial.

In view of these facts, particularly Cannon's observations, it seemed to us important to determine whether trauma *per se* apart from excessive pulmonary ventilation and reduction in the body's content of CO_2 would cause a reduction in the CO_2 capacity of the blood. As Protocols 1 and 2 show, the answer is clearly in the negative. In general the analytical methods employed were similar to those used in the previous papers of this series.

Experiment 1.—Dog, female, 9 kilos. General intestinal trauma. The animal breathed air containing a sufficient percentage of CO_2 to prevent loss due to hyperpnea.

Time.	Condition.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p.m.		per cent	vol. per cent	vol. per cent	mm.
3.20	Normal.		48	49	
3.30	Morphine, 0.18 gm.				
4.30			57	54	122
5.00	CO_2 started.	7.3			130
5.10	Intestinal trauma started.				122
5.40	Intestines very congested.		54	51	100
6.10	" engorged.		54	51	
6.13	Accidental hemorrhage of about 100 cc.				
6.40	Manipulation stopped.	7.6	52	50	78
7.10	Heart stopped. Respiration continued for a minute or two.				

Death in this case was probably due to the large amount of blood which stagnated in the traumatized viscera and to loss of heat. Such a death may be assigned to shock, but it is a shock of a different type from that induced by less trauma when the CO_2 of the body is greatly reduced.

In the following experiments we have employed a method of inducing shock which avoids the objections above pointed out to general visceral trauma. An incision under morphine anesthesia was made in the midline of the abdomen over the stomach barely large enough to admit the hand. The hand carefully wrapped with cotton was then introduced up to the wrist, and the space

Digitized by Google

around the wrist was packed with cotton to prevent loss of heat or the escape of a loop of intestine. The clenched hand was moved rapidly 50 or 60 times a minute so as to bring the knuckles with a downward and forward motion upon the duodenal end of the stomach. The result was vigorous respiration on the part of the subject. Several times there was vomiting. The animal was kept upon a warm plate and there was no fall of rectal temperature. Any consciousness of pain was prevented by the morphine administered.

In Experiment 2 trauma and excessive breathing were produced in this way. The animal was, however, made to breathe

Experiment 2.—Dog, male, 12 kilos. Gastric manipulation, while the animal breathed CO_2 and later without CO_2. Equilibrating air 5.55 per cent CO_2.

Time.	Condition.	Respirations per min.	CO_2 breathed.	Blood CO_2.		Arterial pressure.
				Content.	Capacity.	
p.m.			*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
1.45	Normal.	26		48	50	
2.00	Morphine, 0.24 gm.					
2.15		16				108
2.45		15		67	60	110
2.53	CO_2 started.		6.8			
3.05	Abdominal incision.	19				120
3.15	Gastric manipulation started.	56				120
3.30		54				120
3.45	Vomited.	43	5.8	56	50	130
4.15		44	7.0			128
4.45		37	7.1	53	56	116
4.53	Manipulations stopped.					
5.10	CO_2 stopped.					
5.15		7				
5.30		7	0	41	49	106
6.30		9	0	46	47	106
6.45	Gastric manipulation.	34				108
7.05	Manipulation stopped.	34				
7.15		9		36	36	70
7.30	Dog dead.					44

air containing a sufficient amount of CO_2 to prevent loss. From the protocol it will be seen that after an hour and 38 minutes

Digitized by Google

under these conditions the CO_2 content and the CO_2 capacity of the blood were still practically normal, and arterial pressure had not suffered any considerable fall. Both the CO_2 administration and the manipulation were then stopped for 2 hours. No very marked change followed. Then the manipulation was repeated but without inhalation of CO_2. In 20 minutes the CO_2 content and capacity had fallen to the critical level and arterial pressure was undergoing a decline which ended 25 minutes later in death.

In Experiment 3 the manipulation of the stomach was carried out in the manner above described for 1 hour. The animal did not breathe CO_2. 23 minutes after the termination of this treatment death followed.

Experiment 3.—Dog, female, 9 kilos. Manipulation of stomach. Equilibrating air 5.4 per cent CO_2.

Time.	Condition.	Respirations per min.	Blood CO_2.		Arterial pressure.
			Content.	Capacity.	
p.m.			*vol. per cent*	*vol. per cent*	*mm.*
2.00	Normal.	20	48	49	
3.15	Morphine, 0.19 gm.	10			
3.30		11	61	54	126
3.45	Gastric manipulation started.				
4.00		50	43	52	94
4.15			37	49	
4.30		46	28	38	86
4.45	Manipulation stopped. Reflexes absent.				
5.00		36	27	32	58
5.08	Dog dead.				40

In Experiments 4 and 5 the manipulation of the stomach was carried out until a distinct condition of shock as judged by CO_2 capacity, absence of reflexes, and low arterial pressure was attained. The attempt was then made to reverse these conditions and to restore the animal by causing it to inhale CO_2 in the percentages shown in the protocols. In neither case was there any distinct improvement, and death followed. Autopsy showed little engorgement of the intestines and no hemorrhage, but there was a marked dilatation of the stomach.

Digitized by Google

Experiment 4.—Dog, male, 7½ kilos. Gastric manipulation, followed by an attempt to treat low CO_2 capacity with CO_2. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	CO_2 in air breathed.	Blood CO_2.		Arterial pressure.
				Content.	Capacity.	
			per cent	*vol. per cent*	*vol. per cent*	*mm*
10.00 a.m.	Normal.	22		46	49	
10.05 "	Morphine, 0.15 gm.					
10.45 "		10		58	56	110
10.55 "	Gastric manipulation started.					
11.00 "		64				155
11.15 "		52				134
11.30 "		58				120
11.45 "		46		43	47	115
12.15 "		54		30	33	75
12.25 "	Manipulation stopped. Moribund, no reflexes.					
12.30 "	Started CO_2.	39	7.6	26	27	35
12.45 "		20	7.6	31	26	32
1.00 p.m.		18	7.6	33	29	25
1.15 "		21	7.6	34	30	30
1.28 "	Heart stopped.					

Experiment 5.—Dog, female, 7 kilos. Similar to Experiment 4. Equilibrating air 5.5 per cent CO_2.

Time.	Condition.	Respirations per min.	CO_2 in air breathed.	Blood CO_2.		Arterial pressure.
				Content.	Capacity.	
p.m.			*per cent*	*vol. per cent*	*vol. per cent*	*mm.*
2.15	Normal.	21		50	49	
2.30	Morphine, 0.14 gm.					
3.00		12		59	53	100
3.30	Manipulation started.	47				130
3.45		53				80
4.00		60		39	42	
4.15	Reflexes weak.					40
4.20	Manipulation stopped.					
4.25				34	37	40
4.30	CO_2 started.		8.0			
4.45		30	9.1	41	36	50
5.00			9.7			40
5.15			10.3	46	38	35
5.30			11.0	46	35	25
5.43	Heart stopped.					

Digitized by Google

Evidently beyond the critical point the processes causing lowering of the CO_2 capacity and other ill effects are not reversible. Perhaps at these low levels a true acidosis due to tissue asphyxia resulting from failing circulation sets in, as suggested some years ago in papers on this general subject from this laboratory.[4]

The matter is particularly interesting now in view of the fact that Porter[5] has recently reported beneficial results by causing wounded soldiers in shock to breathe CO_2. In the light of our observations we should expect this treatment to hasten the recovery of those cases whose CO_2 capacity was still above the critical level and who would therefore in all probability have recovered slowly without treatment, but to be only a means of temporarily prolonging life in cases in which the CO_2 capacity had already sunk below the critical level.

In this connection we may mention the fact that some years ago one of us had an opportunity to observe and treat in this way a case of shock. The case was that of a boy who had been run over by a railroad train. One of his feet had been cut off and the other badly mangled. Apparently there had been no considerable hemorrhage. Apparently also there was, in accord with our correspondent quoted in Paper III, at no time agonizing pain. Nevertheless, even when he first came under observation within an hour after the accident the character of the breathing was such as to indicate a distinct tendency toward apnea. In the course of the next few hours during the night there was Cheyne-Stokes breathing. Next morning he was at the point of death at about 9 o'clock, respiration having reached the stage of asphyxial inspiratory gasps. CO_2 gas was administered by holding an inverted glass funnel connected with a tank of CO_2 above his nose. Under such inhalation respiration was restored to a form strikingly like that of normal sleep. There was a marked improvement in the color of the skin and lips and in other vital signs. The treatment was continued for 8 or 9 hours and certainly prolonged life fully to this extent. Nevertheless the circulation gradually failed and death followed.

---

[4] Henderson, Y., *Am. J. Physiol.*, 1908, xxi, 126; 1908–09, xxiii, 345; 1909, xxiv, 66; 1909–10, xxv, 310, 385. Henderson, Y., and Scarbrough, M. McR., *ibid.*, 1910, xxvi, 260. Henderson, *ibid.*, 1910–11, xxvii, 152.

[5] Porter, W. T., *Boston Med. and Surg. J.*, 1917, clxxvii, 326.

Digitized by Google

CONCLUSIONS.

In reports from Cannon on his observations upon shock in wounded soldiers, emphasis has been placed upon the lowering of the alkaline reserve of the blood, upon the fact that under anesthesia and operation a further lowering occurs, and that intravenous injection of alkaline solution has strikingly beneficial effects.

Our experiments show that apart from overventilation of the lungs trauma does not cause a lowering of the CO_2 capacity (alkaline reserve) of the blood, even when by general traumatization and cooling of the abdominal viscera and consequent stagnation of the blood in the injured parts, failure of the circulation and death are induced.

If, on the other hand, no measures are taken to prevent excessive pulmonary ventilation, the overbreathing induced even by localized manipulation of the stomach, while the rest of the viscera are protected, causes lowering of the CO_2 content and CO_2 capacity of the blood, and also of arterial pressure. If the lowering of the CO_2 capacity is pushed beyond the apparently critical level, between 33 and 36 volumes per cent, the disturbance of the vital equilibrium results fatally.

The same duration and degree of localized manipulation administered while the subject is inhaling 6 or 7 per cent of CO_2 does not cause any marked lowering of the CO_2 capacity, arterial pressure, or general vitality.

Below the critical level for the CO_2 capacity, treatment by the administration of 7 to 10 per cent CO_2 in the air breathed does not cause restoration of CO_2 capacity or arterial pressure.

These results are in general harmony with the conception of acapnia as a factor in shock, long advocated in papers from this laboratory.

Further investigations are under way on the therapeutic and prophylactic sides.[6]

The expenses of the investigations reported in this series of papers have been defrayed by the Loomis Medical Research Fund.

[6] Papers dealing with the effects upon the circulation of excess and deficiency of CO_2 will be published shortly in the *Journal of Pharmacology and Experimental Therapeutics*.

Digitized by Google

Digitized by Google

A FOAM INHIBITOR IN THE VAN SLYKE AMINO NITROGEN METHOD.

By H. H. MITCHELL and H. C. ECKSTEIN.

(*From the Department of Animal Husbandry, University of Illinois, Urbana.*)

(Received for publication, January 11, 1918.)

This laboratory has experienced considerable difficulty recently in finding a suitable substance to prevent foaming during the determination of aliphatic amino nitrogen in animal and plant extracts according to the Van Slyke nitrous acid method. Kahlbaum's secondary caprylic alcohol, recommended by Van Slyke, cannot be obtained on the market now, while other preparations we have tried have invariably affected the volume of gas not absorbable by the alkaline permanganate solution. Thus, 0.2 cc.[1] of the secondary caprylic alcohol obtained from castor oil by refluxing with NaOH and subsequently distilling and fractionating,[2] gave 1.00 cc. more of gas not absorbable by alkaline permanganate in an amino nitrogen determination, than was obtained in a duplicate determination in which no alcohol was used. A preparation of caprylic alcohol obtained from Eimer and Amend gave similar results, even when fractionally distilled. We attempted to overcome this effect by shaking the alcohol with glacial acetic acid and a 30 per cent solution of $NaNO_2$ previous to using, and were able in this way to reduce the effect to 0.3 cc. of residual gas. If the alcohol, treated in this manner, was run in with the reagents and shaken with them during the removal of the air from the apparatus, instead of being run in with the sample as is customary, the effect could be reduced still further. Unfortunately, however, this preliminary treatment of the alcohol seemed to deprive it largely if not entirely of its foam-inhibiting character and therefore cannot be recommended. These experiments are interesting in showing that the vitiating effect of these caprylic alcohol preparations on the determination occurs

[1] This amount of foam inhibitor is of course excessive in routine work.

[2] Lee, O. I., *St. Luke's Hosp. Med. and Surg. Rep.*, 1917, iv; abstracted in *Chem. Abst.*, 1917, xi, 2808, 3027.

Digitized by Google

as the result of interactions with the reagents in the decomposition chamber of the apparatus.

We have been able to confirm the conclusion of Van Slyke that amyl alcohol also increases the gas volumes in amino nitrogen determinations, though its effect on the blank is negligible. The excess gas due to the amyl alcohol seems to be roughly proportional to the amount of amino nitrogen in the sample.

Lee's work[2] on foam inhibitors for the urease method of determining urea, in which the solutions are subjected to rapid aeration for the removal of the ammonia formed, lead him to recommend a mixture of 70 per cent phenyl ether and 30 per cent amyl alcohol. He claims that this mixture is more effective than either substance alone or than caprylic alcohol. We have tried this mixture of Lee's in the Van Slyke method, only to find that an effect was produced on the gas volumes obtained in the course of actual determinations, though no effect on the blank could be detected. Phenyl ether alone, however, proved to be without effect, as the following experiments indicate.

10 cc. of an asparagine solution produced in two determinations 16.70 and 16.80 cc. of gas. With 0.2 cc. of phenyl ether and 10 cc. of this solution, two other determinations under the same conditions of temperature and pressure gave 16.69 and 16.72 cc. of gas. Two other determinations in which the phenyl ether was replaced by Lee's foam-inhibiting mixture gave 17.18 and 17.19 cc. of gas. Evidently this effect is produced by the amyl alcohol.

In another experiment a solution of Kahlbaum's leucine was used. 10 cc. portions of this solution gave 9.70 and 9.73 cc. of gas in two determinations in which no foam inhibitor was used. With 0.2 cc. of phenyl ether, two more determinations under the same conditions gave 9.75 and 9.73 cc. of gas.

Phenyl ether thus appears to have no effect on the volume of gas produced in a Van Slyke determination, even when present in excess. Furthermore, according to our experience it is a very effective foam inhibitor, more effective than either amyl or secondary caprylic alcohol. Certain solutions that have foamed excessively with amyl alcohol added in amounts as large as 0.5 cc., during a Van Slyke determination, can now be analyzed with the addition of only two drops of phenyl ether, with very little if any foaming, even when the apparatus is shaken over 300 times per minute.

Digitized by Google

Besides being an effective foam inhibitor with no detrimental effects, phenyl ether may be recommended for this purpose because it can be readily synthesized at comparatively low cost. We have used the method of Ullmann and Sponagel[3] with satisfactory results.

A convenient procedure is as follows: Into a 1.5 liter round-bottomed flask are weighed 560 gm. of bromobenzene, 420 gm. of phenol, 221 gm. of KOH, and 3.5 gm. of copper-bronze. This mixture is heated on an oil bath at 210–230°C. for about 2.5 hours under a reflux condenser. We have used as a condenser a 30 inch glass tube of ¾ inch bore, topped by a water condenser of equal length. Even with this arrangement it is difficult if not impossible to prevent some loss of bromobenzene, especially during the early stages of heating. The mixture is then distilled with steam. The distillate is separated in a separatory funnel and the heavy oil at the bottom fractionally distilled. The boiling point of phenyl ether is 252.3°C. We have taken off fractions from 244–261°C. for use.

The yield may be increased greatly, at least if the steam distillation has not been carried to completion,[4] by extracting the residue from the steam distillation, in small portions, with ether, three washings for each portion being sufficient. The ether extracts are then distilled. A careful and repeated fractionation of the oil from the steam distillation and of the material extracted by ether from the residue is advantageous. After several fractionations it will be found that most of the material falls into two fractions; namely, from 150–168°, and from 244–261°C., the former fraction being bromobenzene and the latter phenyl ether. From the quantities of chemicals given above, we have obtained 287 gm. of phenyl ether, representing a yield of 47.5 per cent of the theoretical, figured on the basis of 560 gm. of bromobenzene, or of 73 per cent when from the amount of bromobenzene taken that recovered in the final fractional distillation (200 gm., boiling from 150–168°) is deducted. The relatively large amount of bromobenzene thus recovered would suggest that the time of refluxing at 210–230° could be lengthened considerably to advantage.

Phenyl ether melts at 28°C., but when in the liquid state it may be supercooled considerably without solidifying. We have observed no solidification of our product at temperatures above 20°C.

[3] Ullmann, F., and Sponagel, P., *Ber. chem. Ges.*, 1905, xxxviii, 2211.

[4] Complete steam distillation of the above mixture would consume too much time. We continued the process for about 4 hours, at which time the condensed water was still somewhat cloudy. Our experience suggests that the steam distillation may well be omitted and the product recovered from the mixture after refluxing, by ether extraction entirely.

Digitized by Google

Digitized by Google

STUDIES OF THE BLOOD FAT AND LIPOIDS OF THE DOG BEFORE AND AFTER THE PRODUCTION OF EXPERIMENTAL ANEMIA.

By HARRY DUBIN.

(From the John Herr Musser Department of Research Medicine, University of Pennsylvania, Philadelphia.)

(Received for publication, January 5, 1918.)

By the use of the nephelometric and colorimetric methods of Bloor,[1] blood fats have been estimated in one dog and the results show that in trypanosome anemia, the total fats are increased while the lecithin and cholesterol are decreased. These results are in accord with the findings of Bloor[2] in pernicious anemia associated with carcinoma of the stomach.

Fats and Lipoids of Dog's Blood before and after Infection with Trypanosoma equiperdum.

Period.	Date.	Total fat.		Lecithin.		Cholesterol.		Remarks.
		Whole blood.	Plasma.	Whole blood.	Plasma.	Whole blood.	Plasma.	
	1917	*gm.**	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
Normal.	Apr. 26	0.55	0.56	0.43	0.40	0.25	0.30	Apr. 24, hemoglobin, 99.
	" 28	0.52	0.53	0.40	0.41	0.29	0.26	
	" 30	0.50	0.52	0.45	0.32	0.25	0.27	
	May 2	0.53	0.57	0.37	0.35	0.22	0.27	Infected May 4.
Anemic.	May 16	0.68	0.69	0.38	0.30	0.22	0.19	May 18, hemoglobin, 56.
	" 18	0.70	0.75	0.29	0.27	0.20	0.23	
	" 22	0.71	0.78	0.31	0.25	0.14	0.12	

* All figures represent gm. per 100 cc. of blood.

[1] Bloor, W. R., Studies on blood fat. II. Fat absorption and the blood lipoids, *J. Biol. Chem.*, 1915, xxiii, 317; The determination of cholesterol in blood, 1916, xxiv, 227; Fat assimilation, xxiv, 447.

[2] Bloor, The distribution of the lipoids ("fat") in human blood. *J. Biol. Chem.*, 1916, xxv. 577.

Digitized by Google

Digitized by Google

ANIMAL CALORIMETRY.

THE INFLUENCE OF MECHANICAL WORK UPON PROTEIN METABOLISM DURING THE HEIGHT OF MEAT DIGESTION IN THE DOG.

FOURTEENTH PAPER.

BY H. V. ATKINSON.

(From the Physiological Laboratory, Cornell University Medical College, New York City.)

(Received for publication, January 16, 1918.)

In the last paper of this series[1] the heat production of a dog was measured, while he was running, during the 4th and 5th hours after the ingestion of 750 gm. of meat. As the dog would not retain urine during a period of exercise, it was necessary to assume, in making the calculations of the metabolism of the period, that the protein metabolism was the same as that which obtained in the same dog at rest after giving the same quantity of meat. However, this assumption might have been false because, on the one hand, the largely increased general blood flow might have furnished the gut with a notably increased volume of blood, thereby accelerating the absorption of amino-acids, or, on the other hand, one might conceive that the demands of the working muscles for blood might so decrease the supply of blood available for the intestines that a marked retardation in the absorption process might have ensued.

A fox-terrier female weighing about 10.5 kilos was found to be willing to eat at most 600 gm. of meat daily at one meal, and upon this quantity the animal was maintained throughout the experiment. As it was found that the animal would not hold urine while running on the treadmill for a period of 1 hour, the period of active exercise was reduced to half an hour, though the urine was collected for the whole hour.

A preliminary series of experiments showed that the maximal quantity of urinary nitrogen was eliminated during the 5th hour after administering meat, as appears in the following table.

[1] Anderson, R. J., and Lusk, G., Animal calorimetry, XIII, *J. Biol. Chem.*, 1917, xxxii, 421.

Digitized by Google

TABLE I.

The Hourly Elimination of Nitrogen during the 4th, 5th, and 6th Hours after Administering 600 Gm. of Meat to a Resting Dog.

Date.	N in urine per hr.		
	4th hr.	5th hr.	6th hr.
	gm.	gm.	gm.
Oct. 4, 1917	1.00	1.08	1.05
" 5 "	1.01	1.04	1.06
" 13 "	1.06	1.11	1.11
" 14 "	1.01	1.13	1.02
Dec. 29 "	1.07	1.05	1.08
Jan. 2, 1918	1.05	1.07	1.09
Average	1.04	1.08	1.07

The dog was then caused to run on the treadmill for 30 minutes in four experiments during the beginning of the 5th hour, and then in two later experiments during the end of the 5th hour. The following results were obtained.

TABLE II.

The Influence of a Run of Half an Hour during the 5th Hour after the Administration of 600 Gm. of Meat upon the Nitrogen Elimination of That Hour Contrasted with That of the Previous and Subsequent Hours When No Work Was Done.

Date.	N in urine per hr.		
	4th hr.	5th hr.	6th hr.
	gm.	gm.	gm.
Oct. 25, 1917	1.07	1.09*	—
Nov. 3 "	1.02	1.08†	1.06
" 6 "	1.09	1.12†	1.11
" 8 "	1.05	1.05†	1.04
Dec. 31 "	1.06	1.07†	1.08
Jan. 3, 1918	1.04	1.03†	1.10
Average	1.05	1.07	1.07

* Distance traveled, 2,090 meters = 1.30 miles.
† Distance traveled, 2,125 meters = 1.32 miles.

It is clear, on comparison of Tables I and II, that *mechanical work has no influence on the hourly rate of absorption of protein or on the intensity of the hourly metabolism of protein in a dog which has been given meat in large quantity.*

Digitized by Google

NOTES ON THE DIRECT DETERMINATION OF UREA AND AMMONIA IN PLACENTA TISSUE.

By FREDERICK S. HAMMETT.

(*From the Department of Anatomy of Harvard Medical School, Boston.*)

(Received for publication, January 14, 1918.)

INTRODUCTION.

Certain questions as to method of analysis have come up during the course of some work on a study of the urea content of placenta tissue. From the point of view of routine work the method of Sumner (1) comes nearest to the analytical ideal of combined accuracy, speed, and minimum of manipulation. Inasmuch as the results herein reported have been obtained by following his procedure no recapitulation is necessary, any alterations being considered in detail in the text.

Urease.

The application of the urea-splitting enzyme of the soy bean, discovered by Tacheuchi (2) and intensively studied by Van Slyke and Cullen (3, 4), to the determination of urea in blood and tissues was first made by Marshall (5). I have used, however, an extract, prepared in Folin's laboratory, of the jack bean whose urease content was recorded by Mateer and Marshall (6, 7). This extract has proved satisfactory in every way.

Potassium Carbonate.

Fiske (8) and Van Slyke and Cullen (3) advocate the use of highly concentrated solutions of potassium carbonate for the liberation of ammonia. It is more advantageous, however, with placenta tissue to use the solid salt, inasmuch as the volume of the contents of the test-tube is already large due to the water used in rinsing down and suspending the pulped tissue. Considerable variations in the quantity employed are recorded in the literature ranging from 5 (1) to 12 (9) gm. of solid carbonate. Van Slyke and Cullen (3) determined that the use of less than 1 gm. of carbonate for each 2 cc. of solution necessitates a longer aeration.

I have found that 5 gm. of solid carbonate is amply sufficient

Digitized by Google

to cause complete liberation of the ammonia from as much as 20 cc. of solution when aerated vigorously for 30 minutes. In view of the increasing difficulty in obtaining potassium salts as well as their increasing cost and the necessity for conservation, minimal amounts consistent with adequate action are indicated. Table I shows the results of a series of comparative tests when using 5 and 10 gm. of solid carbonate, other conditions being equal.

In view of the fact that large variations in the amounts of NH_3 in the tissue suspensions might cause appreciable variations in the efficiency of the amounts of carbonate used, advantage was

TABLE I.

Relative Quantity of NH_3 Liberated from Placenta Tissue Suspensions in 15 Cc. of Solution When Using 5 and 10 Gm. of K_2CO_3 and Strong Aeration for 30 Minutes.

NH_3 in 100 gm. of placenta tissue.	
5 gm. K_2CO_3.	10 gm. K_2CO_3.
mg.	*mg.*
22.8	23.0
16.3	15.8
13.9	14.4
12.3	11.9
11.4	11.6
7.6	7.6
4.9	4.8
4.8	4.6
4.7	4.7

taken of the NH_3-forming ability of urease to produce in some of the suspensions higher NH_3 values. Accordingly, some of the figures represent not only the ammonia as such of the tissue, but also the ammonia liberated from the urea.

It is quite plain that 5 gm. of potassium carbonate is a sufficient quantity for general use.

Time and Vigor in Aeration.

If two samples of the same tissue differing in weight are aerated slowly for 2 minutes and then vigorously at the rate of 5 liters a minute for the remainder of 30 minutes, the amount of ammonia blown over is proportional to the amount of tissue taken for

Digitized by Google

analysis. This lends additional support to the contention of Van Slyke and Cullen (3) that longer periods of aeration as advanced by Fiske (8) and others are unnecessary. Table II gives a few of the results demonstrating this point.

Repeated tests have been unable to demonstrate the carrying over into the receiving flask of any alkaline spray from the aerated mixture mentioned by Sumner (1) as a reason for diminished vigor in aeration. A comparison of the traps used by Sumner and those of this laboratory shows a markedly higher effi-

TABLE II.

NH_3 Recovered from Varying Amounts of Placenta Tissue When Uniformly Aerated for 30 Minutes.

Specimen No.	Weight of tissue.	100 Gm. of tissue.		
		0.01 N NH_3	0.01 N HCl	NH_3
	gm.	*cc.*	*cc.*	*mg.*
10	9.95	8.60	10	2.4
	7.17	8.97	10	2.4
46	6.65	8.40	10	4.1
	5.12	8.76	10	4.1
29	7.29	6.00	10	9.3
	5.97	6.71	10	9.4
46 a	7.13	5.80	20	33.8
	4.45	11.12	20	33.9

ciency in this respect for the latter. Foaming is easily prevented in the aerated mixture by the addition of 1 cc. of a solution containing equal parts of amyl alcohol, toluene, and ethyl alcohol. The use of long-necked 100 cc. flasks prevents loss by spattering, and the foaming in these containers is reduced to a minimum by the addition of a few drops of the above mixture from time to time.

Factors Influencing the Ammonia Fraction in the Urea Determination.

The quantity of urea in any given sample is usually determined by the difference between the ammonia already present and the total ammonia blown over after the action of urease. Having

Digitized by Google

standardized conditions of aeration, enzyme action, and quantity of carbonate used, the changes in the ammonia content of the tissue during standing while undergoing fermentation introduce a considerable error.

Folin and Denis (10) early pointed out that the ammonia content of drawn blood increased if the sample was allowed to stand. Although Mateer and Marshall (6) have definitely directed that in the determination of urea by the urease method ammonia determinations need to be made simultaneously and similarly, only excluding the urease preparation, various published reports have failed to mention or consider ammonia determinations; Slemons and Morriss (11) have even gone so far as to state that such analyses yield insignificant results.

TABLE III.

Increase in the Quantity of NH_3 Recoverable from Placenta Tissue after Standing Half an Hour.

NH_3 in 100 gm. of tissue.	
Immediate.	After ½ hr.
mg.	*mg.*
6.5	7.4
5.9	6.8
2.3	2.9
3.0	4.1
1.7	2.8
1.2	1.9

In order to determine whether or not there was any marked difference between the ammonia obtained by immediate aeration and that given off from the placenta tissue after it had stood for ½ hour in suspension in the test-tube, a series of tests was made, the results of which are found in Table III.

It is evident that increases in the ammonia content of the tissues ranging from 14 to 85 per cent may occur, which alone indicates the necessity of allowing the tissue from which the ammonia values are to be determined, as a basis of the analysis for urea, to stand under similar conditions as do those samples in which the urea is undergoing splitting by the urease.

The results reported in this work are based on parallel determinations and have an average variation of 2 per cent.

Digitized by Google

SUMMARY.

1. In the determination of ammonia or urea in placenta tissue the use of solid potassium carbonate offers opportunity to maintain a minimum volume of the aerated mixture.

2. 5 gm. of the solid potassium carbonate are sufficiently effective in liberating the ammonia present in amounts of tissue ranging from 4 to 10 gm. and suspended in 10 to 15 cc. of water.

3. 2 minutes of mild aeration followed by 28 of vigorous passage of air completely drives over all the ammonia present in the amounts of tissue ordinarily used for analysis.

4. The use of a mixture of equal parts of amyl alcohol, toluene, and ethyl alcohol prevents foaming in test-tube and flask.

5. The ammonia content of tissue increases during standing for ½ hour.

6. In the direct determination of urea in tissues by means of the urease method it is imperative that simultaneous analyses be made for the ammonia fraction. The tissue samples from which the ammonia values are to be obtained must be allowed to digest for the same length of time and under the same conditions as do those specimens from which the urea is determined.

Through the courtesy of Professor Otto Folin the work reported in this paper was carried on in the Biochemical Laboratories of the Harvard Medical School.

BIBLIOGRAPHY.

1. Sumner, J. B., *J. Biol. Chem.*, 1916, xxvii, 95.
2. Tacheuchi, *J. Coll. Agric., Tokyo*, 1909, i, 1.
3. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, 1914, xix, 211.
4. Van Slyke and Cullen, *J. Biol. Chem.*, 1916, xxiv, 117.
5. Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 487.
6. Mateer, J. G., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1916, xxiv, p. xxx.
7. Mateer and Marshall, *J. Biol. Chem.*, 1916, xxv, 297.
8. Fiske, C. H., *J. Biol. Chem.*, 1915, xxiii, 455.
9. Hoagland, R., and Mansfield, C. M., *J. Biol. Chem.*, 1917, xxxi, 487.
10. Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.
11. Slemons, J. M., and Morriss, W. H., *Bull. Johns Hopkins Hosp.*, 1916, xxvii. 343.

Digitized by Google

Digitized by Google

COMPARISON OF THE GLUCOSE AND CHOLESTEROL CONTENT OF THE BLOOD.

By F. H. McCRUDDEN and C. S. SARGENT.

(From the Robert B. Brigham Hospital, Boston.)

(Received for publication, January 4, 1918.)

The association of hyperglycemia and hypercholesterolemia in diabetes[1,2] and of hypoglycemia and hypocholesterolemia in progressive muscular dystrophy,[3,4] pointing to some kind of physiological relationship between glucose and cholesterol, suggested that there might be a parallelism between the amounts of the two substances in the blood. But this appears not to be the case (Table I and Chart 1).

Glucose was determined by the method of Lewis and Benedict,[5] and cholesterol by the method of Autenrieth and Funk.[6] Analyses were made in duplicate or triplicate.

---

[1] Bloor, W., The lipoids ("fat") of the blood in diabetes, *J. Biol. Chem.*, 1916, xxvi, 417.

[2] Joslin, E. P., Bloor, W. R., and Gray, H., The blood lipoids in diabetes, *J. Am. Med. Assn.*, 1917, lxix, 375.

[3] McCrudden, F. H., and Sargent, C. S., Hypoglycemia and progressive muscular dystrophy, *Arch. Int. Med.*, 1916, xvii, 465.

[4] McCrudden and Sargent, Chemical changes in the blood and urine in progressive muscular dystrophy, progressive muscular atrophy, and myasthenia gravis, *Arch. Int. Med.*, 1918, xxi, 252.

[5] Lewis and Benedict's method (*J. Biol. Chem.*, 1915, xx, 61) as modified by Myers and Fine (Myers, V. C., and Fine, M. S., Chemical Composition of the Blood in Health and Disease, New York, 1915.)

[6] Authenrieth, W., and Funk, A., Ueber kolorimetrische Bestimmungsmethoden: die Bestimmung des Gesamtcholesterins im Blute und in Organen, *Münch. med. Woch.*, 1913, lx, 1243.

Digitized by Google

TABLE I.

Case.	Condition.	Glucose.	Cholesterol.
		mg. per 1 gm.	*mg. per 1 gm.*
1	Chronic arthritis	0.086	1.32
2	" "	0.0862	1.50
3	Paralysis agitans	0.088	1.45
4	Chronic arthritis	0.088	2.01
5	" "	0.088	2.09
6	" "	0.0896	1.45
7	Psoriasis	0.090	1.53
8	Pernicious vomiting	0.092	2.22
9	Chronic arthritis	0.098	1.41
10	Bone tuberculosis	0.100	2.07
11	Chronic arthritis	0.101	1.43
12	" "	0.103	1.94
13	" "	0.104	1.55
14	" "	0.106	1.69
15	" "	0.106	1.88
16	Normal	0.109	2.09
17	Chronic arthritis	0.111	2.11
18	Normal	0.115	1.70
19	Chronic arthritis	0.117	2.26
20	Multiple sclerosis	0.120	1.53
21	Arteriosclerosis	0.120	1.54
22	"	0.123	1.59
23	Chronic nephritis	0.123	2.30
24	" arthritis	0.124	1.57
25	" "	0.125	1.55
26	" endocarditis	0.127	1.69
27	" arthritis	0.128	1.29
28	Normal	0.129	1.71
29	Chronic arthritis	0.131	1.45
30	" nephritis	0.133	1.17
31	Paralysis agitans	0.137	2.01
32	Chronic arthritis	0.145	2.35
33	" nephritis	0.147	1.96
34	Lues	0.140	1.71
35	Progressive muscular atrophy	0.179	1.70
36	Chronic nephritis	0.204	1.75

Digitized by Google

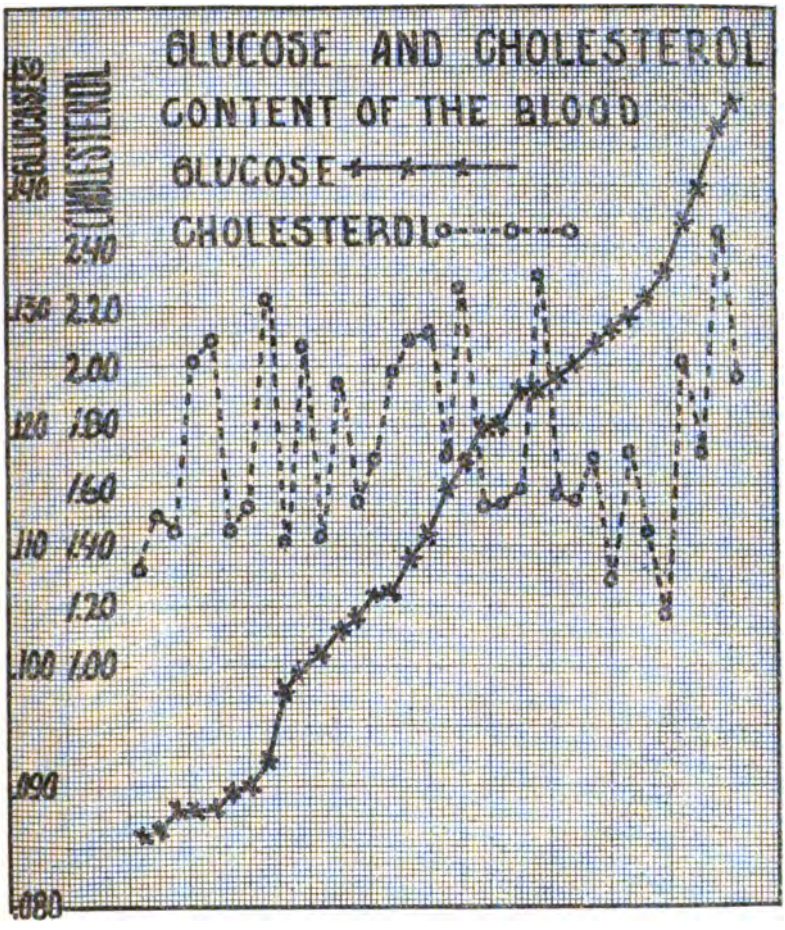


CHART 1. The crosses connected by a full line show the glucose content in per cent of the blood of thirty-four patients (arranged in order of magnitude).

The circles connected by a broken line show the cholesterol content of the blood, also in per cent, in the same cases.

Digitized by Google

Digitized by Google

THE ANALYSIS OF MILK SECRETED BY A SUCKLING DOE KID.

By R. L. HILL.

(From the Department of Biochemistry of the Maryland Agricultural Experiment Station, College Park.)

(Received for publication, January 18, 1918.)

The literature written on the secretion of colostrum contains various theories advanced to account for the appearance of colostrum instead of normal milk immediately following parturition. Some authorities believe that imperfect functional activity of the mammary glands at this time is responsible for the secretion of a fluid so different in composition and consistency from normal milk. Still others are of the opinion that the newly born require a food of the nature of colostrum for the first few feedings before they receive milk; that the colostrum was provided by nature to compensate for this need.

In the newly born there is sometimes a secretion from the mammary glands known as "witches milk." This fluid has a qualitative resemblance to milk but differs widely quantitatively from normal milk. It is often secreted at birth but disappears soon afterward. There have been instances in which young animals have been reported as secreting milk usually as a result of mechanical manipulation of the udder or by being suckled. Whether the first milk so secreted was normal milk, colostrum, or of the nature of "witches milk" is not, to my knowledge, recorded.

On May 2, 1917, a one-half breed Saanen milk goat at this Station was delivered of twin doe kids. One kid was sold when about 3 months old. The other kid appeared perfectly normal but was observed to have an exceptionally well developed udder when about only 2 months old. Its udder was not manipulated, suckled, or treated in any way to stimulate its development. When 4 months and 4 days old, on September 6, 1917, its udder

Digitized by Google

had the appearance of containing milk. Upon examination it was found to contain milk, which was withdrawn and analyzed. A yield of 120 cc. was obtained and proved to be perfectly normal milk, as is shown in the table. Since this secretion was of a spontaneous nature and had not been initiated by any applied stimulus it was decided to remove the milk at intervals several days apart in order to avoid any mechanical stimulation due to repeated milkings. From the following table the normal character of all the milk secreted can be clearly seen.

Chemical Analyses of Milk from a Virgin Kid.

Date.	Fat.	Protein.	Ash.	Total solids.	Sugar.	Milk.	Remarks.
1917	per cent	per cent	per cent	per cent	per cent	cc.	
Sept. 6.....	4.20	3.60	0.84	13.32	4.68	110	Kid, 4 mos., 4 days old.
" 15.....	4.40	3.58	0.82	13.44	4.61	90	
" 22, 23, 24.	4.32	3.53	0.84	13.22	4.53	—	Composite.
" 29.....	4.78	3.36	0.81	13.52	4.57	90	
Oct. 3.....	4.52	3.32	0.75	13.56	4.97	110	
" 5.....	5.60	3.43	0.80	14.20	4.37	75	
" 13.....	5.41	3.51	—	—	—	130	
" 29.....	4.82	—	—	—	—	185	
Nov. 11.....	—	—	—	—	—	240	
" 21.....	—	—	—	—	—	295	
" 29.....	—	—	—	—	—	175	
Dec. 2.....	—	—	—	—	—	100	
" 11.....	4.17	3.94	0.78	13.09	4.20	175	
" 17.....	4.70	4.08	0.80	13.87	4.29	125	

The physical character of the milk is practically the same as normal goat's milk. The curd obtained by the use of rennin or pepsin is, however, much finer and softer than that obtained from any other of the goat's milk examined. The milk was substituted in the diet of an 11 months' old baby for normal goat's milk without any detectable difference either in the appetite or physical condition of the baby. The milk cannot be distinguished by odor or flavor from any other sample of goat's milk. It has a very low bacterial content and keeps for an exceptionally long period. The relative percentage of casein and albumin is practically identical with normal goat's milk. The work is being continued, and any

Digitized by Google

changes in the composition or character of the milk will be noted as they appear.

SUMMARY AND CONCLUSIONS.

A 4 months' old virgin doe kid spontaneously commenced to secrete milk having all the characteristics, properties, and chemical composition of the milk secreted by its mother and other goats under observation. At no time, from the earliest secretion of milk to the present time, has there been any colostrum secreted. This would indicate that the secretion of colostrum is associated with and possibly is produced by the cessation of pregnancy and may not occur in lactation not associated with pregnancy.

Digitized by Google

Digitized by Google

THE CATALASE CONTENT OF ASCARIS SUUM,* WITH A SUGGESTION AS TO ITS RÔLE IN PROTECTING PARASITES AGAINST THE DIGESTIVE ENZYMES OF THEIR HOSTS.

By THOMAS BYRD MAGATH.

(From the Department of Anatomy, College of Medicine, University of Illinois, Chicago.)

(Received for publication, January 8, 1918.)

Many explanations have been offered as to why gastric and intestinal parasites are not digested by their hosts. A brief survey and discussion of many theories can be found in text-books on physiology, for this question resolves itself into the time-worn query, Why is living tissue not digested? The theory which has perhaps gained most attention is that anliferments or antienzymes protect living matter, and this theory has been used by Weinland (1903) to explain the resistance of intestinal parasites to the destructive influence of digestive enzymes. Upon rubbing up *Ascaris* into a pulp and extracting with alcohol he obtained a substance which he termed *Antifermente* because he claimed that this substance protected the proteins from the proteolytic action of pepsin and trypsin. On the basis of his own interpretation this theory was open to criticism because he had to kill the worms to obtain the substance, and it was argued that if the antiferment existed after death it should protect the dead worms as well as the living. More recent work on the antienzymes has offered very good explanations for these phenomena, but it is not the intention to discuss these intricate facts.

In recent times Burge and Burge (1915, *a*) have advanced another theory which might be termed an "oxidation theory," and they have used this not only to explain the resistance of intestinal parasites to the digestive action of their hosts, but maintain that living cells protect themselves from the destructive enzymes by means of the oxidation processes going on in them. It has been shown that the mucosa of the stomach and intestines possesses intense oxidative properties, and Burge (1914) has shown that the digestive enzymes are very easy to oxidize and are then inactive. By applying these facts to intestinal parasites he was able to demonstrate that living worms are not digested while dead worms are, and if the dead ones are permeated with nascent oxygen they withstand

* It is not unlikely that *Ascaris suum* and *Ascaris lumbricoides* are identical.

Digitized by Google

the attack of the digestive enzymes. It should be noted that he killed the worms by electrolysis, a method by which enzymes may be destroyed (Burge and Burge, 1915, *b*).

In addition to these interesting facts another group of phenomena has accumulated. It has been shown (Burge, 1916) that the catalase content of muscles is highest in those in which more work is done, *i.e.* in which more oxidation is taking place; that during starvation the catalase content of the fat and muscles, with the exception of the heart, is less than normal (Burge and Neill, 1917); that the catalase content of the liver, heart, and blood is decreased in phosphorus poisoning and that the tissues are autolyzed in the same proportions that they lose catalase (Burge, 1917).

It occurred to the author that if this theory was valid and the presence of oxidative processes in the intestinal parasites protected them from digestion, then the body walls of these parasites should yield comparatively large amounts of catalase, if the amount of catalase in the tissue was an indication of the oxidative processes. The common *Ascaris*, a roundworm, from the hog was used in a series of experiments to determine the amount and distribution of catalase in the body.

The method used was one adapted from ordinary gas analysis. It seems to have advantages over the one used by Burge (1916) in that the gas may be collected under standard conditions, the apparatus is easier to handle, is neater, and perhaps more accurate, although it must be borne in mind that the determination is not an absolute one but comparative. The text-figure shows the apparatus. The material was washed with a 0.75 per cent sodium chloride solution and finely chopped up, weighed in a crucible, and introduced into the bottle C in which had been put 25 cc. of one-half diluted commercial hydrogen peroxide. The pinch-cock E was opened and the bottle stoppered as indicated in the figure, connecting the bottle to the burette, which was filled with water. The stop-cock B was then opened and the water in the burette D leveled to the zero mark by raising or lowering the bulb A. When this was done the pinch-cock was closed, the crucible upset in the hydrogen peroxide, and the bottle C was shaken for 10 minutes. At the end of that time the stop-cock B was closed and the water in the leveling bulb brought to the level of the water in the burette and the reading made. This was corrected for 760 mm. pressure and 25°C. It is obvious

Digitized by Google

that the amounts of gas determined by this method will be less than those made according to the Burge method, and Tables II and III indicate this in the case of frog tissue, run at the same time on tissue taken from the same frog, which had been washed free from blood by running 0.75 per cent sodium chloride through the blood system. It will be seen that the ratio between my

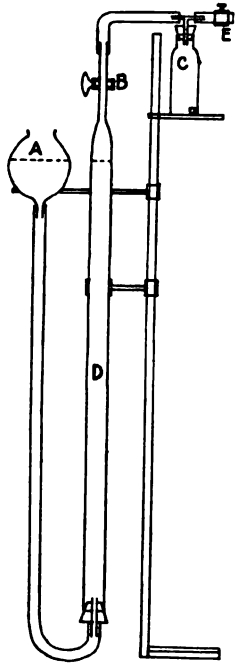


FIG. 1.

method and Burge's is 1: 1.52 in this case. A Schiff's azotometer has been used for catalase determinations (Dox, 1910).

By the method described determinations of the catalase content of the whole worm, the body wall, body fluid, and visceral organs were made. It should be recalled that the body wall of these worms consists of an outer, inert, non-cellular cuticula, a thin layer of subcuticula, and inside of this a single layer of muscle cells. The visceral organs consist in adult females, which

Digitized by Google

were the only ones used, almost entirely of the reproductive organs, in which countless numbers of embryos are rapidly being formed and hence very rapid oxidation must be taking place.

From Table I the following facts were obtained: The whole worms yield 15 cc. of oxygen per gm., the body wall 13 cc., the

TABLE I.*

Oxygen per Gm. of Ascaris suum.

Ascaris suum.	Amount used.	Sample.						Average.
		1	2	3	4	5	6	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Whole worm.....	1	16	14	17	15	14	16	15
Body wall.....	1	10	13	16				13
Body fluid.....	1	7	7	7				7
Visceral organs.....	1	23	26	30				26
Total.....								15

* In all the determinations in the tables 25 cc. of hydrogen peroxide were used and the material was shaken in this for 10 minutes. The number of cc. of oxygen liberated was then read.

TABLE II.

(*Author's Method.*)

Oxygen per Gm. of Rana pipiens.

Rana pipiens.	Amount used.	Sample.			Average.
		1	2	2	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*
Leg muscles.....	1	24	22	23	23
Ovary.....	1	130	120	123	124

TABLE III.

(*Burge's Method.*)

Oxygen per Gm. of Rana pipiens.

Rana pipiens.	Amount used.	Sample.			Average.
		1	2	3	
	gm.	*cc.*	*cc.*	*cc.*	*cc.*
Leg muscles.....	1	43	46	42	43
Ovary.....	1	190	189	185	188

Digitized by Google

body fluid 7 cc., and the visceral organs 26 cc. The average of the last three items run separately yielded the same amount as the total worms, thus furnishing a check on the determinations. The cuticula, when freed from the other two layers of the body wall, does not liberate oxygen from hydrogen peroxide but weighs one-fourth the total amount of the wall; hence it will be seen that the other two layers involved really yielded 17 cc. of oxygen per gm.

While these amounts are very small as compared with those obtained from free living animals it must be remembered that these parasites are very sluggish and their activities are greatly curtailed by the limited amount of oxygen in their environment. However, when these results are compared with the case of the frog in Table II, an interesting observation can be made. A gram of muscle and subcuticula from the *Ascaris* yielded about five-eighths as much oxygen as did the reproductive organs, while in the frog the most active muscles in the body yielded only about one-fifth the amount yielded by the ovaries of that animal. In other words, the sluggish and inactive muscles of the *Ascaris* have more than three times the amount of catalase in them as the most active muscles of a very active vertebrate, when they are compared with the reproductive organs of the same animals respectively from which they came. At the present time this seems to be the most fair way to compare the two cases. It remains for someone to show quantitatively how much of this catalase is necessary to carry on the functions of nutrition, motion, etc., and finally that the amount left over is sufficient and does provide the necessary amount of oxygen to protect the worm from the enzymes of its host. The work presented in this paper, while not final, indicates, to the mind of the author, a possible connection between the two statements made by Burge, that intestinal worms are protected from enzymic action by oxidative processes, and that catalase is in some way responsible for the oxidative processes in animals other than parasites. It is hoped that this contribution will serve as a stimulus for others to undertake investigations along this line.

Digitized by Google

CONCLUSIONS.

1. Catalase may be better determined by the new method given in this paper than by the method used by Burge.

2. There is five-eighths as much catalase in the body wall of *Ascaris suum* as in the visceral organs, and one-fourth as much in the body fluid as in the visceral organs.

3. There is three times more catalase in the body wall of *Ascaris suum* than in the leg muscles of *Rana pipiens*, if one uses the amount of the catalase in the reproductive organs of each form as the units of measurement.

4. On the basis of this last statement it can be assumed that there is more than enough catalase in the body wall of this parasitic worm for its metabolic and locomotory functions, and hence it is possible that this excess is used to liberate oxygen for protecting the parasite against the digestive enzymes of its host, if Burge's theory be true.

BIBLIOGRAPHY.

Burge, W. E., Relation between the amount of catalase in the different muscles of the body and the amount of work done by these muscles, *Am. J. Physiol.*, 1916, xli, 153.

Burge, The effect of phosphorus poisoning on the catalase content of the tissues, *Am. J. Physiol.*, 1917, xliii, 545.

Burge, W. E., and Burge, E. L., The rôle of nascent oxygen in regulating the activities of enzymes in animals and plants, *Am. J. Physiol.*, 1914, xxxiv, 140.

Burge and Burge, The protection of parasites in the digestive tract against the action of the digestive enzymes, *J. Parasitol.*, 1915, *a*, i, 179.

Burge and Burge, The rate of oxidation of enzymes and their corresponding pro-enzymes, *Am. J. Physiol.*, 1915, *b*, xxxvii, 462.

Burge, W. E., and Neill, A. J., The effect of starvation on the catalase content of the tissues, *Am. J. Physiol.*, 1917, xliii, 58.

Dox, A. W., The catalase of molds, *J. Am. Chem. Soc.*, 1910, xxxii, 1357.

Weinland, E., Ueber Antifermente, I and II, *Z. Biol.*, 1903, xliv, 1, 45.

Digitized by Google

THE FATTY ACIDS IN HUMAN BLOOD IN NORMAL AND PATHOLOGICAL CONDITIONS.

By FRANK A. CSONKA.

(*From the Laboratory of Dr. J. P. McKelvy, Pittsburgh.*)

(Received for publication, January 2, 1918.)

INTRODUCTION.

As has been shown in previous researches the fatty acids in human blood exist as glycerides, cholesterol esters, soaps, or as free acids; they are also represented as a radical of lecithin and the phosphatide groups. Their content varies in continued starvation, fat absorption, anesthesia, certain pathological conditions, etc., as recently reported and confirmed in a series of papers by Bloor.[1] In normal individuals when care is taken to avoid the above conditions the amount of fatty acids in the blood is fairly constant. But little, if any attention has been given to the quality of the fatty acids, and especially to the proportion of saturated to unsaturated fatty acids as well as the degree of unsaturation.

That there is a difference in the quality of the fatty acids of the whole human body is shown by the fact that the fats called interstitial, depot, and organ fats, each absorb iodine in different proportions, thus signifying that the unsaturated part of the fatty acids varies in the different tissues. Jaeckle[2] found an iodine number of 62 to 73 for human (interstitial) fat, and 70 to 80 per cent of the fatty acids present as glycerides were oleic acid. Hartley's[3] analysis of liver fat shows the presence of higher unsaturated fatty acids than oleic acid as indicated by the iodine number of 165 to 175.

[1] Bloor, W. R., *J. Biol. Chem.*, 1913-14, xvi, 517; 1914, xix, 1. Bloor, W. R., and MacPherson, D. J., *ibid.*, 1917, xxxi, 79. Bloor, *ibid.*, 1917. xxxi, 575.

[2] Jaeckle, H., *Z. physiol. Chem.*, 1902, xxxvi, 53.

[3] Hartley, P., *J. Physiol.*, 1909, xxxviii, 353.

Digitized by Google

From the point of view of hemolytic action, we are especially interested in the unsaturated part of the fatty acids, since it has been shown by Noguchi[4] and Faust and Tallquist[5] that the higher saturated acids, palmitic and stearic, are not hemolytic either as such or as their soaps. On the other hand, the unsaturated fatty acids, as oleic acid, have a strong hemolytic action, and it has been demonstrated by Faust and Tallquist that this acid is the cause of the anemia in *Bothriocephalus latus* infection.[6] Further, Lamar[7] has shown an existing relation between the degree of unsaturation and lytic action as he was able to show that the sodium soap of linoleic acid dissolved pneumococci more rapidly in higher dilutions than sodium oleate. McPhedran,[8] working with red blood cells, was unable to demonstrate any difference in the lytic action of linoleic and oleic acids as Lamar had claimed in the case of bacteria, and concluded that there was no relationship between the lytic action and degree of unsaturation.

In my previous paper,[9] I reported that the iodine number of fatty acids of the blood in cases of pernicious anemia was not exceptionally high; but, as I stated, the matter needed further investigation, as the material used for iodine determinations was a mixture of saturated and unsaturated fatty acids; so that this finding had no other meaning than that unsaturated fatty acids were present. The mere presence of unsaturated fatty acids cannot be considered the primary cause of toxic hemolysis, since such acids exist in normal human blood, as shown below. It is therefore necessary to look further for the causative factor.

[4] Noguchi, H., *J. Exp. Med.*, 1906, viii, 87.

[5] Faust, E. S., and Tallquist, T. W., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 367.

[6] The cholesterol ester of oleic acid is hemolytic but not so strongly so as oleic acid itself. Since they demonstrated in the saponified ether extract of *Bothriocephalus latus* both oleic acid and cholesterol and were unable to show the presence of glycerol, they concluded that the oleic acid was present as a cholesterol ester without attempting to isolate it as such.

[7] Lamar, R. V., *J. Exp. Med.*, 1911, xiv, 256.

[8] McPhedran, W. F., *J. Exp. Med.*, 1913, xviii, 527.

[9] Csonka, F. A., *J. Biol. Chem.*, 1916, xxiv, 431.

Digitized by Google

Methods.

I.

The saponification of the alcohol-ether extract was preferred to the direct saponification of whole blood because of the inconvenience of working with such large quantities, and the large volumes required to saponify in alcoholic KOH medium in a concentration sufficient to be sure that the esters of cholesterol are also split.

Blood was taken from the vein before breakfast.[10] A few crystals of potassium oxalate were added to prevent clotting. 75 to 125 cc. of blood were measured with a pipette and run slowly into 250 to 400 cc. of 95 per cent alcohol with constant stirring. After the protein matter had settled it was filtered through a Buchner funnel by suction. The blood proteins were put into a large extraction shell and extracted by absolute alcohol for 24 hours and again by petroleum ether for 24 hours in a Soxhlet apparatus. The filtrate from the blood protein as well as the absolute alcohol extract was evaporated on the water bath to dryness. It was redissolved in the petroleum ether extract, filtered through cotton, measured, and about an eighth of it was used for the determination of the fat and lipoid content. From the remainder the petroleum ether was distilled off, the residue saponified, and the fatty acid determined according to the method of Gephart and Csonka.[11]

The first step in the separation of the unsaturated from the saturated fatty acids was to convert them into their lead soaps. These were extracted by ether, according to the method of Varrentrapp, which dissolves the lead soaps of the unsaturated fatty acids. This method is not strictly quantitative, but it is the best at present devised for the separation of unsaturated from saturated fatty acid in general.[12]

[10] I wish to thank Drs. C. C. Hartman and W. T. Mitchell, Jr., for collecting the blood samples used in this work.

[11] Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

[12] To demonstrate how this method worked in my hands I give one of the preliminary analyses made of Merck's oleic acid labeled as highly purified. (A), 0.1700 gm. of oleic acid gave 0.1490 gm. of unsaturated fatty acids; yield 87.6 per cent. (B), 0.1080 gm. of oleic acid gave 0.0950 gm. of unsaturated fatty acids; yield 87.9 per cent. A third sample, where 0.1635 gm. of oleic acid was saponified first, gave 0.1558 gm. of unsaturated fatty acids; yield 95.3 per cent.

Digitized by Google

TABLE 1.

Fatty Acids in 100 Cc. of Blood.

Case.	Sex.	Age.	Diagnosis.	Fat + lipoids.	Iodine No. on 100 gm. fat + lipoids.	Fatty acids.			Iodine No. on 100 gm. unsaturated fatty acids.	Hemoglobin (Sahli).	Red blood corpuscles.
						Total.	Unsaturated.				
		yrs.		*gm.*		*gm.*	*gm.*	*per cent of total*		*per cent*	
1. W. T. M.	M.	28	Normal.	0.790	73.7	0.345	0.168	48.8	84.2	98	5,520,000
2. F. A. C.	"	29	"	0.779	60.8	0.300	0.146	48.7	76.3	95	5,216,000
3. C. H. G.	"	30	"	0.755	67.2	0.300	0.136	45.5	92.1	105	6,984,000
4. C. P. B.	"	50	"	0.615	68.6	0.260	0.136	52.4	77.8	—	—
5. E. C.	"	21	"	0.619	—	0.313	0.136	43.4	105.9	—	—
6. K.	F.	24	"	0.627	59.8	0.263	0.137	52.0	88.8	90	4,200,000
7. O. S.	M.	28	Hemolytic jaundice 16 mos. after splenectomy.	0.839	62.0	0.240	0.137	57.3	142.4	63	3,200,000
8. L.	F.	41	Pernicious anemia.	—	—	0.286	0.160	55.9	94.8	15	680,000
9. S. M.	M.	46	" "	0.509	72.8	0.282	0.157	55.5	101.0	26	928,000
10. A. B.	"	29	Secondary anemia, jaundice.	0.564	75.3	0.241	0.141	58.5	107.8	35	3,690,000
11. McK.	"	56	" "	0.685	73.1	0.292	0.163	55.8	71.8	58	3,580,000
12. C.	"	17	Myelogenous leukemia.	0.355	90.1	0.290	0.140	48.3	110.9	15	800,000
13. S. M.	"	50	Cerebral hemorrhage.	0.955	57.9	0.453	0.318	70.2	92.8	105	5,640,000
14. O. H.	"	39	Chronic constipation.	0.777	88.7	0.362	0.255	70.4	111.5	100	5,008,000
15. N.	"	48	" nephritis.	0.890	78.6	0.370	0.252	68.2	136.6	68	4,440,000
16. F.	"	31	Myocardial insufficiency, chronic nephritis.	0.720	61.0	0.252	0.157	62.4	78.3	85	4,420 000
17. D.	"	30	Chronic nephritis.	0.818	67.3	0.333	0.154	46.3	89.8	94	4,328,000
18. H.	"	46	Diabetes mellitus.	0.699	75.9	0.307	0.173	56.5	86.0	90	5,040,000
19. S.	F.	58	" "	0.823	78.2	0.393	0.211	53.8	101.2	83	5,136,000

Digitized by Google

At the end of the titration of the fatty acids by the method of Gephart and Csonka[11] the fatty acids are present as potassium soaps in a mixture of petroleum ether and alcohol. A portion of the blood cholesterol is present in this same solution. To remove the cholesterol the solution is made alkaline, to insure separation more alcohol is added, and extracted twice with petroleum ether. The alkaline solution containing the fatty acids as soaps is transferred to a large beaker, evaporated on the water bath, the residue dissolved in warm distilled water, neutralized by acetic acid, and the fatty acids are precipitated as lead soaps by the addition of an excess of lead acetate solution. The soaps are then filtered off and dried in vacuum. The ether-soluble lead soaps are decomposed by the addition of hydrochloric acid; the free unsaturated fatty acids, after the ether is distilled off, are dried in vacuum, weighed, and saved for the determination of the iodine number according to Hübl.

Since the iodine number of the unsaturated fatty acids in several cases as shown in Table I is higher than that of oleic acid, the

TABLE II.

Case	3	9	10	14	18	19
Iodine No.	117.2	101.1	129.6	136.9	96.4	108.6
Unsaturated fatty acids of alcohol-soluble barium soaps, gm. in 100 cc. of blood	0.035	0.136	0.054	0.165	0.075	0.084

question of the direct evidence of the presence of other or higher unsaturated fatty acids arose in the latter part of this work. Therefore, the unsaturated fatty acids were dissolved in absolute alcohol neutralized by alcoholic KOH and the oleic acid was partly precipitated as barium oleate, placed in a refrigerator for a few hours, and then filtered. The unsaturated fatty acids of the alcohol-soluble barium soaps (Table II) were liberated by hydrochloric acid, extracted by ether, and after the ether was evaporated off the residue was dried, weighed, and used for the determination of the iodine number.

I was especially careful to avoid any alteration or destruction of the unsaturated fatty acids which may result from the use of excessive heat. The saponification and extraction flask described in the previous communication[9] was used to advantage in this work.

Digitized by Google

II.

A review of the literature has shown that in the determination of blood fats the extraction followed by saponification of the extract was to be preferred to the direct saponification. The former method was introduced by Shimidzu[13] who tried to apply the Kumagawa-Suto direct saponification, but found lower results than when he had first extracted the blood with alcohol and then saponified the extract. Berczeler[14] confirmed Shimidzu's claim and recently Rosenthal and Trowbridge[15] discussing the methods of fat determination concluded also that in blood Shimidzu's alcoholic extractions should precede saponification. As a control for the blood fat determinations in the present work it was thought advisable to run duplicate determinations according to the Gephart-Csonka[11] method. To saponify in alcoholic KOH medium

TABLE III.

Gm. of Fatty Acids in 100 Cc. of Blood.

Case	2	3	6	9	14	16	18
Extraction and saponification	0.300	0.300	0.263	0.282	0.362	0.252	0.307
Gephart-Csonka method	0.325	0.326	0.271	0.318	0.355	0.268	0.428

in a concentration sufficient to be sure that also the more resistant esters such as cholesterol esters are split with certainty, only 5 cc. of blood were used.

The results in Table III verify the applicability of the Gephart-Csonka method for blood in cases where we are interested only in the absolute values, with the advantage also of avoiding the time-consuming extraction. The above results show further that the accuracy was not limited by using such small amounts of blood; the higher results were expected, as the extraction is never complete, especially in case such large amounts of blood are extracted.

The method as applied to blood is as follows: To 5 cc. of blood measured into the author's saponification and extraction flask 20 cc. of alcohol

[13] Shimidzu, Y., *Biochem. Z.*, 1910, xxviii, 237.
[14] Berczeler, L., *Biochem. Z.*, 1912, xliv, 193.
[15] Rosenthal, H., and Trowbridge, P. F., *J. Biol. Chem.*, 1915, xx, 711.

Digitized by Google

(95 per cent) and 4 gm. of stick potassium hydroxide are added. The flask is immersed in a boiling water bath and its contents are boiled under a reflux condenser for 1 hour, it is then cooled, and 20 cc. of 20 per cent HCl are added in small portions to free the fatty acids, cooling the flask after each addition of the acid. The flask is filled to the constriction with distilled water, 50 cc. of ether are added, the flask is closed with the stopper, and shaken in a rotary manner for a few minutes. After the ether layer has separated it is blown by pressure into a separating funnel (250 cc.). The ether extraction is repeated twice with 50 cc. portions, collecting the portions in the separating funnel. After each extraction the ether layer is brought to the constriction by adding a little distilled water. The combined ether extract in the separating funnel is washed with several portions of water until the wash water is neutral. The crude ether extract, being free from hydrochloric acid as well as lower fatty acids, is transferred into an Erlenmeyer flask (200 cc.), a glass bead added, and the ether distilled off. The flask containing the residue is placed in the water bath for a few minutes, then in a vacuum desiccator over night to free the residue from moisture. 25 cc. of light, boiling petroleum ether are added, the flask is rotated at frequent intervals, and the solution filtered through a thick plug of fat-free cotton. Wash the flask and filter well with petroleum ether (3 times with 10 cc. portions) and collect the filtrate which is perfectly clear and colorless in an Erlenmeyer flask. Bring the petroleum ether solution to boiling in a water bath and titrate it immediately with 0.04 N alcoholic KOH using phenolphthalein as an indicator; each cc. of 0.04 N KOH is equal to 10.97 mg. of fatty acids. For further details see the original article.[11]

DISCUSSION.

The unsaturated fatty acids are of exogenous or endogenous origin, the latter being derived by synthesis from either carbohydrate or protein. A third source is by the desaturation of food fat and depot fat, which is mobilized for transport. Leathes[16] believes that desaturation is the rôle of the liver in fat metabolism, so we may consider that unsaturated fatty acids occur in normal metabolism.

As Table I shows, the blood of normal individuals contains an average of 0.143 gm. of unsaturated fatty acids per 100 cc. with an average iodine number of 87.5. 48 per cent of the total fatty acids are unsaturated. The iodine number of unsaturated fatty acids varies from 76 to 105 in normal human blood, thus indi-

[16] Leathes, J. B., *Ergebn. Physiol.*, 1909, viii, 356.

Digitized by Google

cating in addition to oleic acid the presence of other both higher and lower unsaturated fatty acids in small amounts.

In pathological conditions, we have found that a generally higher proportion of unsaturated fatty acids than the normal average occurs, and that the iodine absorption power of the unsaturated fatty acids as well as their absolute amount is higher. We may divide the pathological cases according to their hemoglobin content into two groups: (A) normal hemoglobin, and (B) low hemoglobin.

TABLE IV.

Group A.			Group B.		
Case.	Hemoglobin.	Iodine No.	Case.	Hemoglobin.	Iodine No.
	per cent			*per cent*	
13	105	92.8	7	63	142.4
14	100	111.5	8	15	94.8
16	85	78.3	9	26	101.0
17	94	89.8	10	35	107.8
18	90	86.0	11	58	71.8
19	83	101.2	12	15	110.9
			15	68	136.6

The high iodine numbers representing unsaturated fatty acids with higher degree of unsaturation than oleic acid are found in Group B, but we should not forget that in the same group we deal with an abnormally low red cell count. Munk and Friedenthal[17] found an increase of fat in red corpuscles during fat absorption, and Bloor[18] suggested that most, if not all, fat metabolized through lecithin, and that lecithin formation is a result of red cell activity. In anemia, where the number of red cells is lowered, probably the fat metabolism is disturbed; and as a fact we find the amount of lecithin as well as other lipoid substances generally lower than that in normal human blood. It would seem that these lipoid substances especially require the unsaturated fatty acids as a radical in their formation. The higher iodine value found especially in those cases where the lipoids are low suggests a resultant excess of unsaturated fatty acids and not necessarily an increased desaturation of fatty acids by stimulated liver activity.

[17] Munk, I., and Friedenthal, H., *Centr. Physiol.*, 1901–02, xv, 297.
[18] Bloor, *J. Biol. Chem.*, 1916, xxiv, 447.

Digitized by Google

While we know that this excess of unsaturated fatty acids has a hemolytic action, and no doubt intensifies the hemolysis, yet it cannot be the primary cause, as we found in Cases 3, 13, 14, 19 (Tables I and II) higher unsaturated fatty acids in even larger quantities than in normal blood with normal red blood count and hemoglobin content.

The iodine values of fat plus lipoid substances show small variation in pathological cases as well as in normal cases, and have neither qualitative nor quantitative significance. A higher iodine number does not mean that there are present more highly unsaturated fatty acids than oleic, or that the proportion of unsaturated to saturated is larger.

SUMMARY.

Unsaturated fatty acids are a product of normal metabolism, being present in normal human blood, in which they form 48.0 per cent of the total fatty acids. Judged by their iodine absorption power there are, in addition to oleic acid, other unsaturated fatty acids both higher and lower than oleic, although in small amounts.

The iodine numbers of the unsaturated fatty acids in pathological conditions are generally higher than in normal individuals, especially in cases with low hemoglobin values. As such acids exist in normal human blood as well as in pathological conditions without anemia, it is necessary to look further for the primary cause of toxic hemolysis.

The Gephart-Csonka method for fatty acid determination in feces has been applied to blood in the present work, thus avoiding the time-consuming extraction common to other methods.

Digitized by Google

Digitized by Google

A BIOLOGICAL ANALYSIS OF PELLAGRA-PRODUCING DIETS.

V. THE NATURE OF THE DIETARY DEFICIENCIES OF A DIET DERIVED FROM PEAS, WHEAT FLOUR, AND COTTENSEED OIL.

By E. V. McCOLLUM, N. SIMMONDS, and H. T. PARSONS.

(From the Chemical Laboratory of the School of Hygiene and Public Health of the Johns Hopkins University, Baltimore.)

(Received for publication, January 25, 1918.)

There are described in the literature two types of experimental diets which are reported to have produced, the one in man and the other in dogs, a pathological condition which closely resembles, if indeed it is not actually identical with human pellagra (1, 2). It has long been suspected that pellagra is in some way the sequel to the long continued use of a faulty diet, but there have been various theories as to the manner in which the diet is at fault.

The experiments of Goldberger (1) have rendered the relationship of the diet to the disease extremely probable. Men were restricted during 5½ months to a diet prepared from wheat flour (patent), corn meal, (corn) grits, cornstarch, white polished rice, standard granulated sugar, cane syrup, sweet potatoes, pork fat (fried out of salt pork), cabbage, collards, turnip greens, and coffee. At the end of 5½ months, five of the eleven men thus restricted, showed unmistakable signs of the disease. Goldberger interpreted his results with creditable caution, but decided that, "On the whole, however, the trend of available evidence, strongly suggests that pellagra will prove to be a 'deficiency' disease very closely related to beri-beri" (1).

Voegtlin (3), after a critical examination of the literature, and also from clinical observations, formulated his views concerning the etiology of pellagra as follows:

"From a survey of the clinical and pathological aspects of pellagra, I have arrived at the conclusion that we are dealing with a chronic intoxication. While the agents at work in this intoxication are as yet unknown, I am inclined to believe that toxic substances exist in certain vegetable food, not necessarily spoiled, which if consumed by man over a long period

Digitized by Google

of time, may produce an injurious effect on certain organs of the body. This hypothesis does not rule out the possibility that a dietary deficiency . . . . [vitamines] . . . . may play a rôle in the production and treatment of pellagra."

"One will have to consider very seriously: (1) A deficiency or absence of certain vitamines in the diet. (2) The toxic effect of some substances, as aluminum, which occur in certain vegetable foods. (3) A deficiency of the diet in certain amino-acids."

Chittenden and Underhill (2) produced in dogs a condition which they describe as closely similar to pellagra in man, by feeding a diet consisting of cooked (dried) peas, cracker meal, and cottonseed oil. Their views concerning the number of diseases which fall into the same category with beri-beri, that is in being caused by the lack of specific protective substances, are formulated as follows:

"The absence or deficiency of these substances ('vitamines') may lead to a variety of metabolic disturbances which have been designated by different names as beri-beri, scurvy, pellagra, etc., and which may be grouped together as 'deficiency diseases.'" They concluded from their numerous experimental data that, "From the facts enumerated the conclusion seems tenable that the abnormal state may be referred to a deficiency of some essential dietary constituent or constituents, presumably belonging to the group of hitherto unrecognized but essential components of an adequate diet."

As a result of studies with diets composed of purified foodstuffs supplemented with fats of the growth-promoting and non-growth-promoting classes, and with alcoholic and water extracts of plant tissues (4) and also with the addition of small amounts of various natural foods (5), we were led to the conclusion that there are possible of classification two still unidentified food complexes which are essential in the diet, and we introduced the terms fat-soluble A and water-soluble B to designate them (6). The prefixes "fat-soluble" and "water-soluble" have now become generally adopted by other investigators, although there is still lack of uniformity in the terminology in other respects. There are not less than twenty different terms in the literature of the last 2 or 3 years to designate these two substances, or as a few authors seem to believe, groups of substances of unknown nature.[1]

[1] Recently Emmett and McKim (7) have argued in favor of the existence of a special "vitamine" for maintenance and another or others for growth. We are not able to see in the experimental data which Emmett and McKim present any evidence in support of the interpretation which the authors put upon their data.

Digitized by Google

It is obviously of the greatest importance that we have a correct understanding of the relation of diet to disease, and it is accordingly exceedingly desirable that it be definitely established whether there is in reality but a single essential substance in each of what we term fat-soluble A and water-soluble B. If, as the evidence available indicates, each represents but a single substance, there can be but two deficiency diseases in the sense in which Funk employed this term (8). He used the term "deficiency diseases" to designate a series of four or more syndromes, beri-beri, scurvy, pellagra, and rickets, and expressed the belief that each was due to the lack of a specific protective substance. We have, therefore, directed our efforts to the solution of the problem concerning the number of the unidentified dietary essentials, and have repeatedly expressed the view that there are but two (9).

McCollum and Pitz (10) demonstrated that scurvy in the guinea pig does not belong in the category of "deficiency" diseases, and referred it instead to the agency of microorganisms in the digestive tract. The stagnation of feces in the cecum of the guinea pigs employed as experimental animals was suggested to be the primary cause of the development of the organisms concerned. Hess (11) has recently described experiments which support our conclusions regarding the bacteriological factor in the production of scurvy. He found that freshly pasteurized milk does not produce scurvy in the human infant, whereas the same milk kept 24 hours after the heat treatment may do so. The most plausible explanation for this is the development of pernicious forms of organisms after the pasteurization has checked the lactic acid formers. We hold, therefore, that scurvy has been definitely eliminated from the list of supposed "deficiency" diseases.

In the previous articles of this series, we have analyzed the deficiencies of several seeds, from the dietary standpoint, by suitably planned feeding experiments (12, 13). We have arrived at the conclusion that beri-beri was properly judged by Funk as to the cause of its origin, but that the other diseases which he held to be due to the lack of specific substances from the diet are in reality due to other causes. We have called attention to clinical evidence of the existence in man of cases of xerophthalmia (9), entirely analogous in origin to the same pathological state in rats, brought about by a deficiency of the fat-soluble A in the diet. Xerophthalmia and polyneuritis are abundantly demon-

Digitized by Google

strated to have their origin in the lack of a sufficient amount of the fat-soluble A and water-soluble B respectively in the diet. The experiments described in this paper make it clear that the diet of Chittenden and Underhill is not deficient in the sense that it fails to furnish a sufficient amount of another specific substance which when present protects against the development of the syndrome of pellagra. The deficiencies of their diet are all dependent upon the shortage of the fat-soluble A, the character of the inorganic moiety, and the relatively poor quality of its protein mixture. The experimental demonstration of this fact, provided the interpretation be accepted that their dogs were suffering from a disease analogous to pellagra in man, eliminates a second syndrome, pellagra, from the list of supposed "deficiency" diseases.

On the appearance of Chittenden and Underhill's paper, we were at once convinced that their interpretation of the nature of the deficiencies of their diet could be only partially correct. This conclusion is inevitable in the light of our many studies of all the more important seeds, which are in use as foods for men and animals. Each of these, we have shown, can be supplemented by the addition of inorganic salts, purified protein, and a growth-promoting fat, so as to be dietetically complete (14). It must follow, therefore, that the fat-soluble A is the only unidentified dietary factor of which there is any relative lack in any of the seeds which we have studied. The deficiency of the seeds in fat-soluble A is only relative, not absolute. Each of the seeds appears to contain at least 50 per cent of the requirements of the growing rat for this substance, provided the other factors in the diet are of good quality. A mixture of peas, wheat flour (or crackers), and cottonseed oil, should, in the light of our data with each of the common seeds, be susceptible of fairly accurate appraisal as to its dietetic value. The experiments described in this paper demonstrate the correctness of this view.

Chart 1. Lot 1,915.—The standard food mixtures of peas, patent flour, and cottonseed oil induced no growth in Period 1. In Period 2 the addition of purified protein did not correct the ration so as to produce growth.

Lot 1,916 A shows that a suitable salt addition was not sufficient to render this food mixture capable of supporting growth,

Digitized by Google

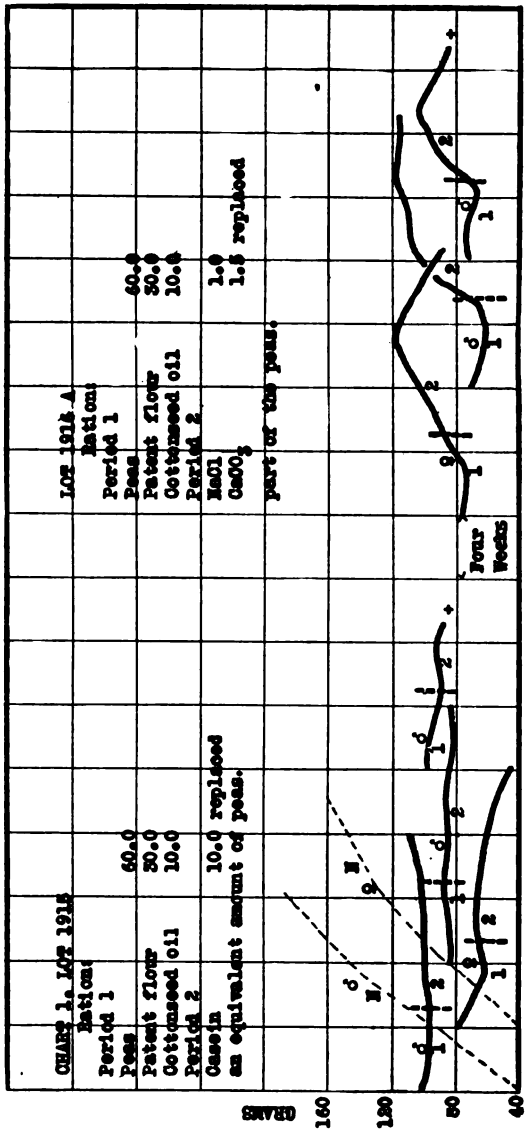


CHART 1.

Digitized by Google

while Lot 1,021, Chart 2, and Lot 1,022, Chart 3, demonstrate that the content of the dietary factor, fat-soluble A, is not the factor limiting growth. No matter how well the proteins of this diet are supplemented with purified protein addition, with or without increasing the content of the diet in fat-soluble A, growth cannot take place unless the elements sodium, chlorine, and calcium are added. We have shown in the first (12) and fourth papers (15) of this series that these are the only inorganic elements which are required to be added to any of the seeds in order to make them dietetically complete, as far as the mineral content is concerned. Compare Chart 1 with Charts 2 and 3.

These groups of rats developed very rough scaly tails, and numerous bleeding points covered the surface. The ears were thickened and on the margins scabs developed. There was a cutaneous horn on the nose of each rat. These signs of pathological changes on the skin are common in our rat colony in animals fed certain types of faulty diets. They are not the unfailing accompaniment of diets which cause debility even of a severe character. We have not yet analyzed with sufficient thoroughness the exact nature of dietary faults which induce these changes.

There was no diarrhea in any of our rats on the diets described in this paper. Neither did we observe infection in the oral and intestinal mucosa.

Lot 1,916 A.—The diet in Period 1 could not support any growth. In Period 2, they received in addition sodium chloride and calcium carbonate. The salt additions induced a slight response with growth for 6 to 8 weeks, followed by decline. Lot 1,021, Chart 2, shows the necessity of adding these salts, if growth is to take place; Lot 1,023, Chart 3, shows that no others are necessary to enable the animals to grow at the normal rate. The inorganic content of this diet is, therefore, the first limiting factor in preventing growth.

The peas were soaked in water, then heated in a sterilizer at 15 pounds' pressure for 1 hour, and dried in a current of air at about 70°C. Gold Medal flour and a commercially bleached cottonseed oil were employed. The animals were given distilled water, and according to our general custom, were furnished water containing iodine in potassium iodide once each week.

Chart 2. Lot 1,916 B.—In Period 1 these rats received the

Digitized by Google

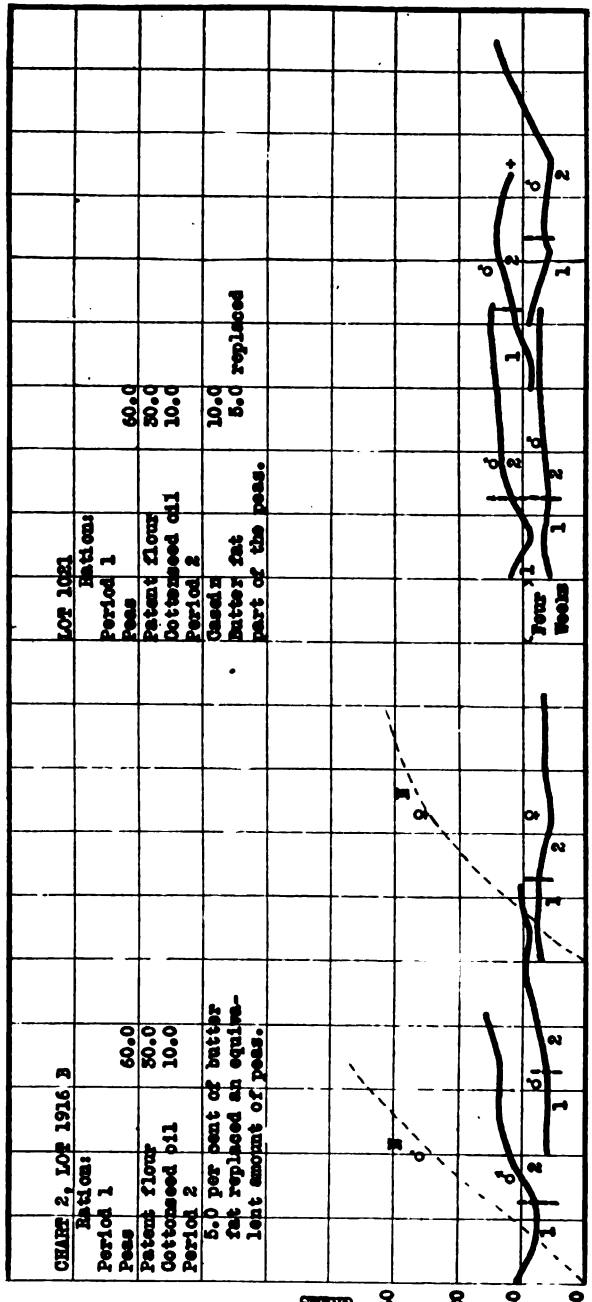


CHART 2.

Digitized by Google

standard mixture and failed to grow. In Period 2 the content of the fat-soluble A was increased by the addition of butter fat, but without noticeable benefit. Chart 1, Lot 1,916 A, showed that the addition of an appropriate inorganic salt mixture, and Lot 1,915 an addition of purified protein did not supplement the mixture of peas, patent flour, and cottonseed oil so as to induce growth. Two purified food additions, one of which is inorganic, are necessary (Chart 3, Lots 1,022 and 1,023). These rats (Lot 1,916 B) showed the same skin lesions on the nose, ears, and tails that were described in Chart 1, Lot 1,915. They differed from the latter in being extraordinarily irritable. They were terror stricken whenever the cage door was opened, and there was always great difficulty in weighing them. Irritability in some degree was characteristic of all the rats on this ration where but one purified food addition was made. Extreme timidity is of common occurrence in our experimental rats on certain types of faulty diet. We are not yet in a position to discuss satisfactorily the exact nature of the causes of this abnormality.

Lot 1,021.—These records illustrate the fact, when interpreted together with Chart 3, that both protein and fat-soluble A (as butter fat) added to the diet of Period 1 fail to make the ration capable of supporting growth. The addition of certain salts (sodium, chlorine, and calcium) is necessary before growth can proceed. These salts alone are, however, not sufficient (Chart 1, Lot 1,916 A). Either the protein must be improved, or the fat-soluble A must be increased in amount, as well as an improvement in the inorganic moiety effected before the food mixture becomes capable of supporting growth (Chart 3, Lots 1,022 and 1,023). It is not apparent from the curves of growth that it is of great importance for some weeks whether the second factor which is improved is protein or fat-soluble A. Growth can take place in either case. Prolonged well-being requires, however, in addition to other supplements, an increase in the content of fat-soluble A in this food mixture.

Chart 3. Lot 1,022.—In Period 1 the animals were fed the standard mixture of peas, patent flour, and cottonseed oil and were unable to grow. In Period 2, the addition of two purified food substances, the simple salt mixture of sodium chloride and calcium carbonate, together with purified casein, induced prompt

Digitized by Google

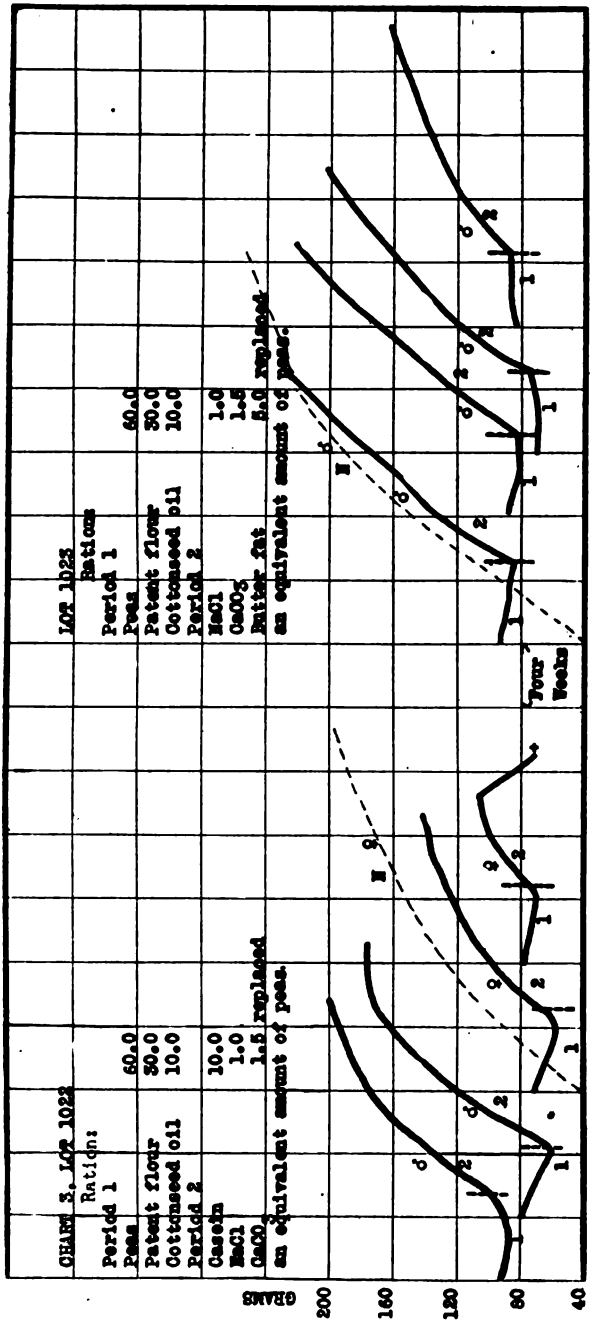


CHART 3.

Digitized by Google

response. Chart 2, Lot 1,021, showed that two purified food additions, protein and fat-soluble A, do not enhance the diet so as to support growth.

The growth of these animals was not so rapid as they are capable of making when the diet is highly satisfactory. This is illustrated by the records of Lot 1,024, Chart 4, when compared with Lots 1,022 and 1,023, Chart 3. In the latter the best rats became stunted before reaching full adult size. These records make it clear that the diet of peas, wheat flour, and cottonseed oil can be supplemented with well known dietary components so as to induce a moderate amount of growth in the rat. It is necessary to conclude, therefore, that the dietary deficiencies of this food mixture, which has induced in dogs symptoms closely resembling pellagra in man, are not in the nature of unidentified "protective" substances, except that there is in some degree a shortage of the content of fat-soluble A. Sodium chloride and calcium carbonate, together with purified protein and fat-soluble A, render the diet practically complete (Chart 4).

Lot 1,023.—These records illustrate the fact that the protein mixture derived from peas and patent flour (12.1 per cent) is adequate for the support of a good rate of growth (Period 2) after a period of stunting for 5 weeks. These records further support the view that the first limiting factor in this food mixture is the quality of the inorganic content (see Charts 1 and 2). These growth curves are not of so steep a gradient, however, as can be secured by the further improvement of the protein content of the diet by the addition of purified casein (Chart 4). A comparison of Charts 3 and 4 makes it evident that more fat-soluble A must be added before the optimum growth can be secured with this ration.

Chart 4. Lot 1,024.—These records show the degree of improvement of a mixture of peas, patent flour, and cottonseed oil, which can be effected by the addition of three recognized dietary factors, protein, salts, and fat-soluble A. Not only is the growth on the diet supplemented in this way nearly at the maximum rate, but three litters of young have been secured.

A mixture of peas, patent flour (crackers), and cottonseed oil induced in dogs a profound state of malnutrition, characterized by sore mouth, sloughing of the mucosa, diarrhea, infection of the

Digitized by Google

mucosa of the intestinal tract, and skin lesions (2). The same diet is shown in this paper to be deficient only with respect to three dietary factors, all of which can be named and but one of which (fat-soluble A) is of chemically unknown nature. This forms a strong argument against the idea that pellagra is a disease in the same category with beri-beri, or the xerophthalmia which was described in the second paper of this series (9). Pel-

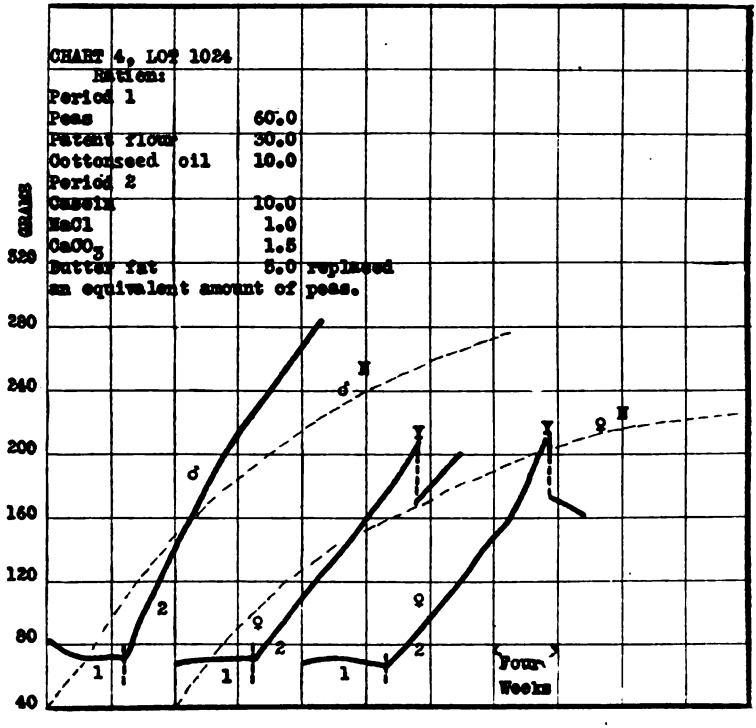


CHART 4.

lagra is primarily, we believe, associated with the unsatisfactory character of three dietary factors as described, and there cannot be a specific protective substance against this syndrome as there is for the other diseases just enumerated. We have elsewhere offered chemical evidence that there is but one water-soluble indispensable food constituent of unknown chemical nature (13). The demonstration that neither scurvy nor pellagra belong in

Digitized by Google

the class of disease for which such protective substances exist, removes the necessity of postulating the existence of more than one indispensable substance in what we term water-soluble B, unless it shall be shown that rickets, the only other disease referable to faulty diet, is comparable in its etiology with beri-beri and xerophthalmia. The experiments reported in this paper support our contention that there are in reality but two "de-

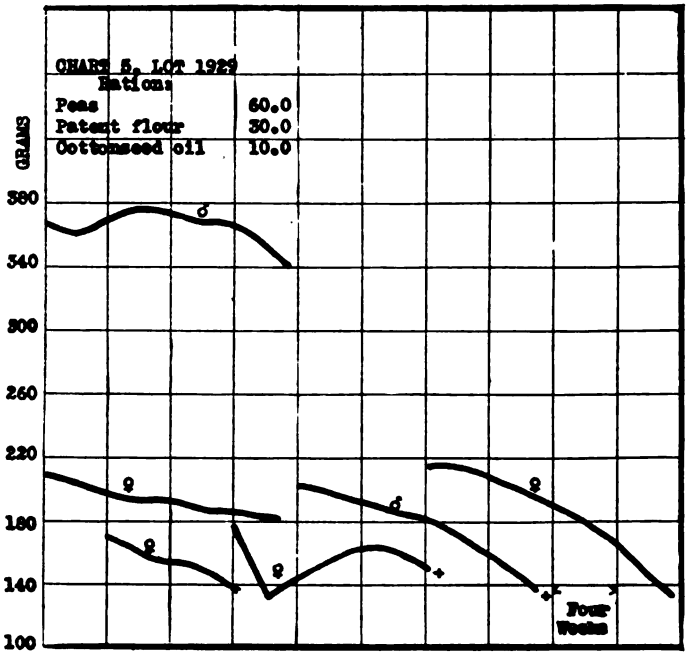


CHART 5.

ficiency" diseases, and but two physiologically indispensable unidentified dietary essentials.

Chart 5. Lot 1,929.—In this chart are shown for comparison with the growth curves of young animals (Charts 1 to 4 inclusive) the curves illustrating the changes in body weight of grown rats confined to the diet of peas, flour, and cottonseed oil. These were at the beginning of the experiment about 1 year old and in excellent condition, and capable, if fed a good ration, of living about 2 years longer. They were restricted to the diet of peas.

Digitized by Google

patent flour, and cottonseed oil, which was employed throughout this work. All suffered steady decline, showing that the diet is not of a character to support either maintenance or growth until properly supplemented. Three of the six animals died at the end of the 8th, 12th, and 15th weeks respectively.

The coats of these animals became rough but there were no thickening of the ears, baldness, soreness of the tails, or cutaneous horns on the noses, such as we have seen to be concomitant with faulty diet. We have not observed therefore, the pathological changes in the mucous surfaces of the alimentary tract nor the skin changes and infections observed by Chittenden and Underhill in dogs. In grown rats, loss of weight and the early appearance of senile characters were the only evidences that this diet was inadequate. In certain groups (Charts 1 and 2) in young rats skin changes were observed.

BIBLIOGRAPHY.

1. Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.
2. Chittenden, R. H., and Underhill, F. P., *Am. J. Physiol.*, 1917, xliv, 13.
3. Voegtlin, C., *J. Am. Med. Assn.*, 1914, lxiii, 1094.
4. McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 231.
5. McCollum, E. V., *J. Am. Med. Assn.*, 1917, lxviii, 1379.
6. McCollum, E. V., and Kennedy, C., *J. Biol. Chem.*, 1916, xxiv, 491.
7. Emmett, A. D., and McKim, L. H., *J. Biol. Chem.*, 1917, xxxii, 409.
8. Funk, C., *Biochem. Bull.*, 1915, iv, 304.
9. McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 181.
10. McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229
11. Hess, A. F., *Am. J. Dis. Child.*, 1917, xiv, 337.
12. McCollum and Simmonds, *J. Biol. Chem.*, 1917, xxxii, 29.
13. McCollum and Simmonds, *J. Biol. Chem.*, 1918, xxxiii, 55.
14. McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 153, 211; 1917, xxix, 521.
15. McCollum and Simmonds, *J. Biol. Chem.*, 1918, xxxiii, 303.

Digitized by Google

Digitized by Google

THE STRUCTURE OF YEAST NUCLEIC ACID.

III. AMMONIA HYDROLYSIS.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 31, 1918.)

Since the tetranucleotide structure of yeast nucleic acid has been generally accepted, the efforts of workers in this field of investigation have been devoted to a search for an explanation of the mode of linkage between individual nucleotides. The theory of the structure of yeast nucleic acid was established through the discovery of methods which yielded on the one hand the individual nucleotides and on the other the pyrimidine nucleotides. For the explanation of the further details in the structure of the substance it was necessary to obtain a fragment of the nucleic acid molecule that would possess a more complex structure than the simple mononucleotides. Attempts in this direction were made by Thannhauser and Dorfmüller[1] and by Walter Jones[2,3,4] and his coworkers. Thannhauser and Dorfmüller reported the discovery of a trinucleotide, Walter Jones with his coworkers the discovery of two dinucleotides. The conclusions of Thannhauser and Dorfmüller were criticised by Jones who very convincingly exposed the weak points in the arguments of Thannhauser.

However, it is now found that the claim of Jones and Germann and Jones and Read to have isolated an adenine-uracil dinucleotide was not well founded. Following exactly the same conditions of analysis as given by these authors a brucine salt was obtained which possessed analytical values required by the dinucleotide. On recrystallization out of 35 per cent ethyl alcohol this brucine

[1] Thannhauser, S. J., and Dorfmüller, G., *Z. physiol. Chem.*, 1915, xcv, 259.

[2] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.

[3] Jones, W., and Germann, H. C., *J. Biol. Chem.*, 1916, xxv, 93.

[4] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 123; xxxi, 39.

Digitized by Google

salt was separated into two fractions, one analyzing for uridinephosphoric acid, the other for adenosinephosphoric acid. Out of 120 gm. of the mixed salt there were obtained 50 gm. of the first portion and 70.0 gm. of the second. The first salt was converted into a barium salt which possessed the crystal form, the optical rotation, and the analytical values of the recently described[5] barium salt of uridinephosphoric acid.

The second fraction was also converted into a barium salt which separated out of a concentrated aqueous solution. However, it was impossible thus far to obtain the salt in crystalline form. Work in this direction is in progress.

It is evident on the basis of this experience that as far as the yeast nucleic acid is concerned the largest fragment obtained up to the present is a mononucleotide. Hence the problem of the mode of linkage between the individual nucleotides still awaits its solution. On the other hand the work done in Jones' laboratory and our recent work have advanced further proof for the tetranucleotide structure of yeast nucleic acid, since on cleavage of the nucleic acid it is now possible to isolate the following three mononucleotides in pure form: guanylic acid, uridinephosphoric acid, and cytidinephosphoric acid; and there is reasonable hope that adenosinephosphoric acid also will be prepared in pure form.

EXPERIMENTAL.

Crude nucleic acid in lots of 100.0 gm. each in 500 cc. of water and 50 cc. of 25 per cent ammonia water were hydrolyzed for 1 hour in an autoclave at 115°C. The reaction product was filtered and to the filtrate an equal volume of 98 per cent alcohol was added. A precipitate was thus formed which was removed by filtration. The filtrate was concentrated to a third of the original volume under diminished pressure (between 12 and 15 mm.), the temperature of the water bath not exceeding 40°C. To the concentrated solution was added again an equal volume of 98 per cent alcohol. To the filtrate a 25 per cent solution of basic lead acetate was added as long as a precipitate formed.

The lead precipitate was ground up in a solution containing 5 per cent lead acetate and filtered. The operation was repeated three times. The washed lead precipitate was suspended in water

[5] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 229.

Digitized by Google

containing barium hydroxide and decomposed by means of hydrogen sulfide. Care was taken to keep the solution slightly on the alkaline side. The excess of hydrogen sulfide was removed by aeration; the solution was then made distinctly alkaline, filtered, again neutralized, and concentrated to a volume of 500 cc. The barium was then removed quantitatively and from the solution the brucine salts were prepared in the usual way.

The analysis of the mixed brucine salts gave the following value.

0.200 gm. substance gave 15.4 cc. N at 18°C. and 747.2 mm.
For cytosine-uracil dinucleotide $C_{19}H_{25}N_7P_2O_{15}.4(C_{23}H_{26}N_2O_4)+14\ H_2O$

	Calculated:	Found:
N	8.46	8.88

This material was recrystallized nine times. The final product gave the following analytical results.

0.200 gm. substance gave 12.4 cc. N at 17°C. and 757.2 mm.
0.1012 " " " 0.1970 gm. CO_2, 0.0582 gm. H_2O, and 0.0058 gm. ash.

	Calculated for $C_{23}H_{26}N_2PO_7+7H_2O$:	Found:
C	53.20	53.08
H	6.51	6.43
N	6.80	7.26

This brucine salt was then converted into the barium salt. The salt crystallized in the manner described in the previous communication. It was redissolved in a small volume of a 10 per cent solution of sulfuric acid. The solution was neutralized with barium hydroxide, filtered, and allowed to crystallize at room temperature. The figure illustrates the crystals.

The substance gave the following analytical data.

0.200 gm. substance required for neutralization 8.92 cc. of 0.1 N acid.
0.1052 " " gave 0.0912 gm. CO_2, 0.0230 gm. H_2O, and 0.0514 gm. ash.

	Calculated for $C_9H_{11}N_2O_8PBa$:	Found:
C	23.50	23.64
H	2.41	2.45
N	6.10	6.24
$Ba_2P_2O_7$	48.97	48.86

The optical rotation of the substance was the following:

$$[\alpha]_D^{20} = \frac{+0.14° \times 100}{1 \times 4} = +3.5°.$$

Digitized by Google

FIG. 1. Barium salt of uridinephosphoric acid.

Digitized by Google

A DETAILED METHOD FOR THE PREPARATION OF HISTIDINE.

By HORRY M. JONES.

(From the Psychopathic Laboratory of Cook County Hospital, Chicago.)

(Received for publication, January 18, 1918.)

The many uses which are being found for histidine in various lines of biological work and the difficulty of obtaining this amino-acid on the market make it necessary for many research laboratories to prepare their own supplies of histidine.

Directions for the preparation of histidine as given in the text-books are usually too meager to lead one to a successful first trial; *i.e.*, a good yield and a pure product. Either a large amount of the product is discarded unawares in one of the precipitates or filtrates, or else the mother liquor is too grossly contaminated with other salts to permit the product to separate in pure crystals. The method given here is offered, however, not as an improvement on the method already in use, but as a more detailed statement of that method, together with reasons for certain apparently insignificant steps in the process which are easily overlooked.

Hemoglobin is the protein of preference as a source of histidine, and ox blood corpuscles, or the so called blood paste,[1] which can be obtained from the slaughter house, are probably the most economical source of histidine.

2 liters of blood paste are placed in a wide-bottomed dish or porcelain-lined pan and to this is added slowly an equal quantity of concentrated HCl. The lumping of the corpuscles into large masses is of no significance, as these lumps will dissolve later. The mixture is then poured into an 8 or 10 liter Jena or Pyrex round-bottomed flask and heated on a sand bath in the fume chamber for about 18 hours. By this time the volume of the mixture will be reduced to about half the original volume; *i.e.*, 2 liters. The biuret test on a neutralized and filtered portion should now be negative. If the biuret test is negative, the flame

[1] "Blood paste" is the concentrated suspension of red blood corpuscles obtained by centrifugating defibrinated ox blood.

Digitized by Google

is removed and the mixture allowed to cool. It is then mixed with an equal volume of saturated sodium carbonate, pouring the acid mixture slowly into the carbonate solution, with thorough mixing. The vessel for this should have a capacity of 40 or 50 liters to allow for the effervescence of such a viscous mass. Finally, after the foam has settled, which requires a few hours, the mixture should be made exactly neutral to litmus paper and filtered. If it has not been carefully neutralized, this filtrate will have a reddish purple muddy color instead of a clear yellow. The filtrate is now made alkaline with 25 gm. of NaOH and boiled to expel ammonia. After an hour of boiling, it may be necessary to add more NaOH to make sure that all the ammonia will be driven off. Any NH_4Cl left in the solution will interfere in all subsequent treatments of the precipitates and filtrates, and will eventually have to be separated from the final product.

After standing for several hours in the cold, the leucine and tyrosine will separate out, and should be filtered off. The filtrate should then be treated, alternately, with concentrated solutions of $HgCl_2$ and of Na_2CO_3, using the Na_2CO_3 to keep the mixture alkaline as more $HgCl_2$ is added. Continue this until a small filtered portion, which should be distinctly alkaline to phenolphthalein, does not produce the heavy voluminous precipitate of mercuric histidine when treated with more of the $HgCl_2$ solution. An opalescent or milky precipitate will continue to form even after all the histidine is precipitated, but should be disregarded. Now filter off the large volume of precipitate and wash by suspending the latter in a 2 liter quantity of distilled water and again filtering. This process is repeated a second time to insure the removal of substances in the original solution, as well as the excess of $HgCl_2$. Suspend the twice washed precipitate in five times its volume of water and, using concentrated HCl, render the latter acid to bromophenol blue (pH = 3). If the dilution is greater than five volumes of water, the same volume of concentrated HCl would be required, but the pH would be higher; and since both the dilution and the pH are more easily measured than the strength of the acid and the volume of precipitate, this method is suggested. At such a dilution, and pH, the mercuric histidine will be redissolved, while a large volume of a dirty brown residue should remain in suspension. The latter is then removed by

Digitized by Google

filtering. The acid filtrate is then treated with a sufficient quantity of concentrated solution of Na_2CO_3 to render the solution distinctly alkaline to phenolphthalein, whereupon the mercuric histidine is reprecipitated as a clean, creamy flocculent mass. The latter is thoroughly washed two or three times by suspending in water and refiltering as before, in order to remove Na_2CO_3 and NaCl.

Finally, it should be suspended in about 600 cc. of water, and H_2S allowed to bubble slowly through the thick suspension, until the mass becomes coal-black. The filtrate from this mass of HgS is then allowed to evaporate at room temperature, whereupon large yellowish crystals of histidine hydrochloride will separate out from the brown, syrupy mother liquor. The yield should be about 15 gm. The crystals are then freed from the brown adhering film of mother liquor, and dried between filters. It should be identified by its melting point (256°), by its crystalline structure, and i s freedom from inorganic salts, proved by incinerating a small portion on platinum foil.

Digitized by Google

Digitized by Google

THE GROWTH OF CHICKENS IN CONFINEMENT.*

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

With the Cooperation of Edna L. Ferry and Alfred J. Wakeman.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

Plates 4 to 6.

(Received for publication, January 10, 1918.)

The possible advantage in the use of chickens as experimental animals in the study of growth and other problems of nutrition has suggested itself to many investigators since the days of Pasteur. These birds can be hatched under artificial conditions; they grow rapidly and therefore may furnish desired evidence more speedily than is the case with most of the domestic animals; and furthermore if the conditions under which chickens continue to grow normally in confinement can be learned, it will be possible to obtain much information of practical use in poultry husbandry.

There is a widespread belief among poultry raisers that young chickens cannot be reared under the artificial conditions of housing and diet which many other experimental animals tolerate without detriment. The current ideas are expressed in the statements that the birds must be kept "on the ground," that they must have exercise, outdoor life, and green food. In a recent report of observations upon the growth of young chickens under laboratory conditions Drummond[1] wrote:

> "There are not many published records of the use of young chickens as the experimental animals in studies upon the factors concerned in growth and nutrition, but, from a reference to those of recent date, the present author was led to believe that one might rely to some considerable extent upon their suitability for such studies."

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Drummond, J. C., *Biochem. J.*, 1916, x, 77.

Digitized by Google

Drummond attempted to rear young chickens in the laboratory without success even when the food supplied was supposed to be the same as that which facilitated growth under conditions of normal environment. He mentions "ruffled appearance" and "weakness in the legs" as symptoms attending the failures. Quoting Drummond further:

"The failure of the normal birds to grow and develop was at first attributed to possible defects in the condition of keeping or feeding. Thus, poultry-keepers sometimes consider that weakness in the legs is due to a deficiency of fresh meat in the diet. It was therefore attempted to rear a batch of young chicks upon normal food, giving them in addition a ration of fresh meat daily. The birds ate the meat greedily, but did not respond to the change, and the effort to raise them was no more successful than before.

It was then thought that if the young birds were reared upon a more natural surface they might thrive more satisfactorily. Accordingly, two batches were reared separately, one being brought up upon a surface of sawdust and coarse sand, scattered on the floor of the cage . . . whilst the other group was provided with a surface of fresh earth and grass."

Post-mortem examination of a large number of these 'normal' chicks did not bring to my notice any particular point of interest. The bodies were without exception greatly emaciated. The organs were usually small and anaemic. During life this apparent poverty in blood was striking. The combs and eyelids of the chicks were peculiarly bloodless, being of a dead white colour, as contrasted with the deep red colour possessed by the combs and eyelids of normal fowls of an equal age."

In conclusion Drummond wrote:

"It is felt that the young chick is not a suitable object upon which to conduct experiments in the study of growth, at any rate, when it is kept under artificial conditions. The difficulty of rearing young fowls indoors is, I believe, realised by poultry-farmers generally, so much so in fact, that it is seldom attempted by them.

An explanation of the cause of the failure has not yet suggested itself. No dietary deficiency can be held as responsible, for the diet supplied to the experimental chicks in the normal batches was in every way similar to that successfully employed to rear chicks in the open air. An attempt to supply one of the conditions, under which fowls are normally reared, was made when fresh earth and grass were given to provide a surface for the young birds to run upon.

They certainly made use of the soil, scratching it over and picking out small insects and other tasty morsels all day long. And yet, despite the fact that the earth was changed frequently, this group of birds reached but a slightly better standard of development than did a similar batch, which had a surface of sawdust and grit to run upon.

Digitized by Google

In the ight of these facts we must admit that there are other factors than an adequate diet, which must be supplied to the growing chick, in order that it may complete its normal growth when under conditions such as are described."

Likewise Funk,[2] in referring to Drummond's conclusion, that even chickens on normal diet do not thrive when kept under laboratory conditions, wrote:

"This fact, of which I am fully aware, has been already emphasized in one of our publications. . . . . Still it also remains a fact that as a result of an inquiry into conditions existing in poultry farms, we are using now a mixture of chicken food, cabbage, and charcoal, the water being entirely replaced by milk, thereby succeeding in diminishing the mortality to a very great extent, in spite of the fact that the birds are kept in small cages."

The experience which we[3] reported in feeding young chicks on selected rations encouraged us to make further attempts to ascertain the conditions under which these birds can be used, if possible, in the laboratory for the study of the factors essential for their nutrition during growth. Two of the chickens, Nos. 5 ♂ and 6 ♂, reported in our earlier paper at the age of 81 days, subsequently attained maximum body weights of over 6 pounds—2,760 and 2,915 gm. respectively at the age of 309 days. Their plumage was well developed, their appearance excellent, and both proved fertile. During a period of almost a year they were kept in cages not over 2 x 2 x 2 feet, were never removed from the laboratory, and never received any green fodder. Photographs taken at the age of 322 days are appended.

During the period of these experiments the protein concentrates fed were corn gluten, cottonseed flour, soy bean flour, and casein. "Protein-free milk" and butter fat were used as sources of "vitamines." Starch, lard, and ferric citrate also entered into the diet. An abundance of water and grit was always furnished.

Since these rations contained no roughage we early trained these chicks to eat the blotting paper with which the bottoms of their cages were lined. The importance of an adequate supply of roughage seems to have been overlooked by those who have at-

[2] Funk, C., *J. Biol. Chem.*, 1916, xxvii, 3, footnote.

[3] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 293.

Digitized by Google

tempted to raise chickens in confinement; and the good effects which appeared to result from the use of paper fiber have led us to continue to feed it in the form of blotting paper or moist paper pulp in our subsequent experiments with chickens. It is surprising with what avidity large quantities of paper were eaten, full grown birds consuming daily a sheet of blotting paper nearly 2 feet square. By supplying this cellulose material the use of the more complex forms of roughage, as grass, straw, etc., was avoided. It is possible that the employment of plenty of oat straw litter by poultrymen has its advantage in the supply of roughage thus furnished rather than in the exercise which it is generally supposed to stimulate.

Out of ten 3 weeks old White Leghorn chicks similarly put on various experimental rations and deprived of all green food, four attained a considerable size. Photographs of two of these are shown at an age of 271 days and weights of 1,520 gm. (3.3 pounds, cockerel) and 1,130 gm. (2.5 pounds, pullet) respectively. The pullet later reached a weight of 1,370 gm. (3 pounds) at an age of 298 days. The cockerel crowed vociferously and the pullet laid two eggs while under observation. Most of the other chickens of this lot, however, developed the characteristic inability to use their legs. Nevertheless many of them continued to gain weight in spite of the fact that they were almost entirely helpless. It is possible that these individuals did not acquire the habit of eating enough of the paper fiber and consequently their digestions became impaired with the subsequent development of "weak legs." On diets where no corn or other material furnishing plant pigments of the xanthophyll or carotin type was present the absence of pigmentation in the epidermal scales on the legs and the ear lobes was noticeable, testifying to the well known fact that the coloring matter in these parts comes from the food. Their combs and wattles were in most cases anemic.

The chicks thus far considered had presumably been reared under outdoor conditions and were 3 to 5 weeks old before they were selected for experiment. Drummond has expressed the conviction that, "The age at which the chick is brought under the influence of these artificial conditions is apparently the chief determining factor in its subsequent development, other conditions being the same." We therefore hatched a large number of White

Digitized by Google

Leghorn chicks in the laboratory. These were fed in groups, in a diversity of ways, our aim being to supply suitable protein, salts, and "vitamines" of both the water-soluble and fat-soluble types. Green food was never used. "Roughage" or "ballast" was furnished in the form of moist paper pulp. Later, many of the chicks ate in addition considerable quantities of the blotting paper with which the bottoms of the cages were covered. The fat-soluble "vitamine" which probably is ordinarily supplied by green food was furnished by a liberal amount of butter fat incorporated in the various rations fed.

It was soon made evident in these newer trials that chicks directly from the incubator can reach several times their initial weight, though fed on the mixtures selected by us, quite as satisfactorily as when the experiments were begun with chicks 3 to 4 weeks old. The rations used contained all of the nutrients which our experience with rats had led us to assume might be essential for growing chicks and in fairly similar proportions. Such changes as were made chiefly involved the physical state of the food and were in accordance with the results of our observation of the habits of these birds.

We are not yet prepared to furnish an interpretation of the unlike growth made by the chicks in the various experiments just referred to. The character of the food mixture used was varied so frequently that we cannot yet discuss this feature in detail. It seems more than fortuitous that the feeding of liberal proportions of gliadin or wheat gluten has had a favorable influence, especially in view of the high esteem in which wheat is held by poultrymen.

In all our different series a considerable number of the chicks developed the familiar "weakness of the legs" that is variously attributed to lack of exercise and other factors incident to indoor conditions. By changes in the diet or by outdoor conditions we have as a rule been unable to cure completely birds thus affected, although some throve fairly well while remaining permanently anatomically defective. Nevertheless a few of the experimental chicks continued to grow under the supposedly adverse conditions of caging and diet, ultimately gaining a weight of more than 1,250 gm. The appearance of two such birds is shown in the photographs taken at the age of 164 days and 1,278 (♀) and 1,267 (♂)

Digitized by Google

gm. of body weight respectively. Chicken 44 ♀ laid eggs after 178 days of age. The male bird, No. 57, reached a stage of development at which it crowed like an adult.

Despite the small proportion of the experimental birds which grew as well as did those here pictured, the success already achieved in the absence of dietary factors hitherto assumed to be essential for the growth of chickens, and also under supposedly adverse conditions of housing, encourages us to believe that all of the essentials for the nutrition and adequate growth of chickens under laboratory conditions can be ascertained, and that these will be controllable in much the same way as has proved possible in the case of other animals. The question of "roughage," suitable salts, proteins, and food hormones needs to be approached from new angles in the case of species that have characteristics of digestion and metabolism and structural requirements somewhat different from those of most mammals.

EXPLANATION OF PLATES.

PLATE 4.

FIGS. 1 and 2. Photographs of Rhode Island Red Chickens 5 ♂ and 6 ♂ (shown at an earlier age in our paper)[3] at the age of 322 days. They weighed between 6 and 7 pounds each.

PLATE 5.

FIGS. 3 and 4. White Leghorn Chickens 11 ♂ and 12 ♀ raised in the laboratory without green food from the age of 3 weeks. The photographs show them at an age of 271 days when they weighed 3.3 and 2.5 pounds respectively.

PLATE 6.

FIGS. 5 and 6. White Leghorn Chickens 44 ♀ and 57 ♂ raised in the laboratory without green food from the time they were a day old. The photographs show them at the age of 164 days when they weighed 2.8 pounds, each.

Digitized by Google

FIG. 3.

FIG. 4.

(Osborne and Mendel: Chickens in confinement.)

Digitized by Google

Digitized by Google

THE EFFECTS OF ELECTROLYTES ON GELATIN AND THEIR BIOLOGICAL SIGNIFICANCE.

II. THE EFFECT OF SALTS ON THE PRECIPITATION OF ACID AND ALKALINE GELATIN BY ALCOHOL. ANTAGONISM.

By W. O. FENN.

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, January 14, 1918.)

In a previous paper[1] the writer described a method for studying the effects of electrolytes on gelatin which was found to possess many advantages. It consisted essentially in adding 95 per cent alcohol to 5 cc. of a gelatin-electrolyte mixture until an opaque precipitate was produced. The number of cc. of alcohol required to produce this precipitate was referred to as the "alcohol number" of gelatin. It can be quickly and accurately determined and is surprisingly sensitive to small variations in the electrolyte content.

The separate effects of acids, alkalies, and salts on the alcohol number of gelatin have already been described. The present paper deals with the effect of salts when combined with acids and alkalies. The effect of mixtures of two salts will form the subject of a later paper.

There is need in biology for a complete knowledge of the equilibrium between salts and acid proteins or alkaline proteins, especially in view of the antagonistic action of salts and acids in their effects on organisms, as described by Loeb,[2] by Osterhout[3] and by others. There is no evidence of antagonistic action between salts and alkalies in biological work, so far as the writer is aware. Instances of antagonism between salts and both alkalies and acids are, however, numerous in investigations on the physical chemistry of protein solutions.

[1] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiii, 279.

[2] Loeb, J., *Arch. ges. Physiol.*, 1899, lxxv, 303. Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

[3] Osterhout, W. J. V., *J. Biol. Chem.*, 1914, xix, 517.

Digitized by Google

Thus Pauli[4] found that anions were most effective in counteracting the effects of acids on the coagulation temperature of egg white. Pauli and Handovsky[5] reported that anions were about equally effective in decreasing the viscosity of alkaline albumin, while the bivalent cations were more effective than the monovalent cations. Pauli[6] concluded that acids and alkalies ionise proteins and that neutral salts prevented this ionization. Hopkins and Savory[7] investigated the action of salts on the Bence-Jones protein from pathological urine. The protein coagulates at 55°C. but dissolves again at 100°C. in the presence of salts. The following relations obtained for this effect of the salts: $CaCl_2 > Na_2SO_4$ in acid solution, $Na_2SO_4 > CaCl_2$ in alkaline solution, and $Na_2SO_4 = CaCl_2$ in neutral solution. Chick and Martin[8] added neutral salts to acid and alkaline gelatin and found a lowering of the H and OH concentration respectively by measurements with a concentration cell. In acid albumin the polyvalent anions were most effective in this respect; in alkaline albumin, the polyvalent cations. Fischer[9] measured the swelling of gelatin in the presence of acid-salt mixtures and found that the effectiveness of the anions in hindering swelling by HCl increased in the order of the lyotropic series, while the effectiveness of the cations increased with the valence. NaCl was found to antagonize both acids and alkalies. The most important step in reaching an understanding of the antagonism between salts and acids was made by Procter[10] who investigated the HCl-NaCl-gelatin equilibrium in order to throw light on the strong dehydrating action of NaCl when used by tanners on the acid tissues of hides.

In the baking industry it is commonly understood that a flour with a high percentage of ash gives a stronger gluten in baking than one with a low percentage. This is attributed to the action of the salts of the flour in antagonizing the weakening effect on the gluten of the acids of fermentation. Hard water is also known to give a stronger gluten for the same reason. Salt is often added to overfermented dough to "neutralize" its acid. This fact has been established by Wood and Hardy.[11]

Other instances of antagonism between acids and salts will be given in a later paper on the precipitation of proteins in mixtures of electrolytes.

From the writer's experiments the following conclusions can be drawn concerning the antagonistic effect of salts on acids and alkalies in gelatin.

[4] Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1907, x, 53.
[5] Pauli, W., and Handovsky, H., *Biochem. Z.*, 1910, xxiv, 239.
[6] Pauli, *Z. Chem. u. Ind. Kolloide*, 1910, vii, 241.
[7] Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.
[8] Chick, H., and Martin, C. J., *Kolloidchem. Beihefte*, 1913, v, 49.
[9] Fischer, M. H., *Kolloid-Z.*, 1915, xvii, 1.
[10] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *ibid.*, 1916, cix, 307.
[11] Wood, T. B., and Hardy, W. B., *Proc. Roy. Soc., Series B.* 1909, lxxxi, 38.

Digitized by Google

1. Salts with monovalent ions (like NaCl) decrease the effect of both acids and alkalies in gelatin.

2. Salts with bivalent (like $CaCl_2$) or trivalent cations decrease the effect of alkalies on gelatin but increase the effect of acid, except in high concentrations of salt or acid, where the effect is decreased.

3. Salts like Na_2SO_4 with bivalent or trivalent anions decrease the effect of acids on gelatin but increase the effect of alkalies, except in high concentrations, when the greater the con-

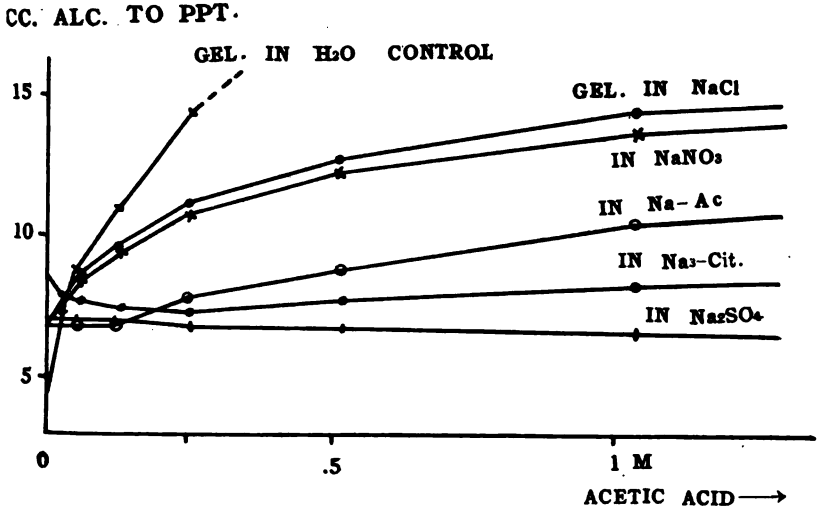


FIG. 1. Curves showing the effect on the alcohol number (ordinates) of increasing concentrations of acetic acid (abscissæ) in gelatin dissolved in water (control) and various sodium salts, all 0.125 M. The sodium salts antagonize the effect of the acetic acid and make precipitation by alcohol possible even in strong acetic acid. (See Table I.)

centration of either salt or alkali, the less the alcohol necessary for precipitation.

The effect of various sodium salts on gelatin plus acetic acid is illustrated in Fig. 1 and Table I. Acetic acid alone entirely prevents the precipitation of gelatin by alcohol except in comparatively low concentrations. In the presence of sodium salts, 0.125 M, however, precipitation by alcohol is possible even in

Digitized by Google

high concentrations of acetic acid. NaCl, for example, has, therefore, decreased the effect of acetic acid on the alcohol number of gelatin. But acetic acid has increased the effect of NaCl. Is this, then, a case of antagonism?

Osterhout has defined two electrolytes as *antagonistic when their combined effect is less than the additive effect*, the additive effect being the effect of the combination if each salt acts inde-

TABLE I.

Antagonism between Acetic Acid and Sodium Salts in Their Action on the Precipitation of Gelatin by Alcohol.

Concentration of acid.	Cc. alcohol to precipitate in gelatin plus					
	H_2O	NaCl	$NaNO_3$	Na acetate.	Na_2SO_4	Na citrate.
M						
4.17	No. ppt.	16.2	14.35	13.1	4.7	9.3
2.08	" "	15.4	15.4	12.0	6.1	9.0
1.04	" "	14.4	13.9	10.4	6.6	8.2
0.52	" "	12.8	12.4	9.0	6.8	7.7
0.26	14.35	11.2	10.9	7.9	6.9	7.4
0.13	10.95	9.75	9.55	7.1	7.0	7.5
0.065	8.85	8.8	8.6	7.0	7.1	7.8
0.032	7.3	7.9	7.8	6.8	7.1	8.0
0.016	6.15					
0.008	5.15					
0.004	4.5					
0.0	4.25	6.85	6.75	7.0	7.1	8.7

The first column shows the concentration of the acetic acid in the gelatin before adding alcohol. The succeeding columns show the number of cc. of 95 per cent alcohol which were necessary to produce a precipitate in 5 cc. of the acetic acid solution plus H_2O-gelatin, NaCl-gelatin, etc. In all cases the salt concentration was kept constant at 0.125 M, while the concentration of acetic acid varied. 3 per cent gelatin B was used throughout, and all the tubes stood over night at 26°C. before adding alcohol. The results of two determinations were averaged for each point. (See Fig. 1.)

pendent of the presence of the other.[12] This is of course the ideal criterion for antagonism. In the case of gelatin, however, it is frequently not easy to apply because the effects of the vari-

[12] For criteria of antagonism in biological work and the determination of the additive effect see Osterhout, *Bot. Gaz.*, 1914, lviii, 178; 1915. lx, 228.

Digitized by Google

ous electrolytes when each is used alone are quite different and their dilution curves are so dissimilar that the additive effects are difficult to determine. When the additive effect is not known, it is safe to say that *antagonism exists if the observed effect is less than that produced by the most strongly acting electrolyte* in the absence of the others (and in the same concentration in which it exists in the combination). When the observed effect is greater than this, there may still be some antagonism but it is very slight. This criterion will, therefore, be used throughout this paper in determining whether or not two electrolytes are antagonistic in their effects.

Returning to Fig. 1, the conclusion may be drawn that acetic acid is antagonized by sodium salts, the bivalent SO_4 ion and the trivalent citrate ion being most effective, and the acetate, which, like the citrate, yields an alkaline solution by hydrolysis, being more effective than the nitrate or chloride.

This experiment (Fig. 1) was designed to elucidate by means of gelatin an experiment of Loeb,[13] where 0.125 M sodium salts was found to prevent the penetration of acetic acid into the egg of *Fundulus*. The order of effectiveness found by Loeb was SO_4>acetate, Cl>NO_3.

The relations of Na_2SO_4 to acid and alkaline gelatin are illustrated in Fig. 2. In this experiment the effect of increasing concentrations of Na_2SO_4 on gelatin dissolved in HCl, NaOH, and water was determined by the alcohol method. The effect of Na_2SO_4 on gelatin plus HCl is found to pass through a minimum. This minimum is probably an isoelectric point due to the neutralization of the effect of the H ions by the SO_4 anions. The proportions of SO_4 to H at this point are as 100: 30. Antagonism is shown by the fact that the combined effect of Na_2SO_4 and HCl at the minimum is less than that of the HCl alone. *Na_2SO_4 and HCl are, therefore, antagonistic in their effects on gelatin,* at least in certain concentrations.

The effect of Na_2SO_4 on gelatin plus NaOH is seen (Fig. 2 and Table II) to pass through a maximum and subsequently fall off. The effect of Na_2SO_4 alone is shown in the dotted line. At no point is the combined effect of Na_2SO_4 and NaOH less than the effect of either alone. *Therefore in low concentrations at least*

[13] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139.

Digitized by Google

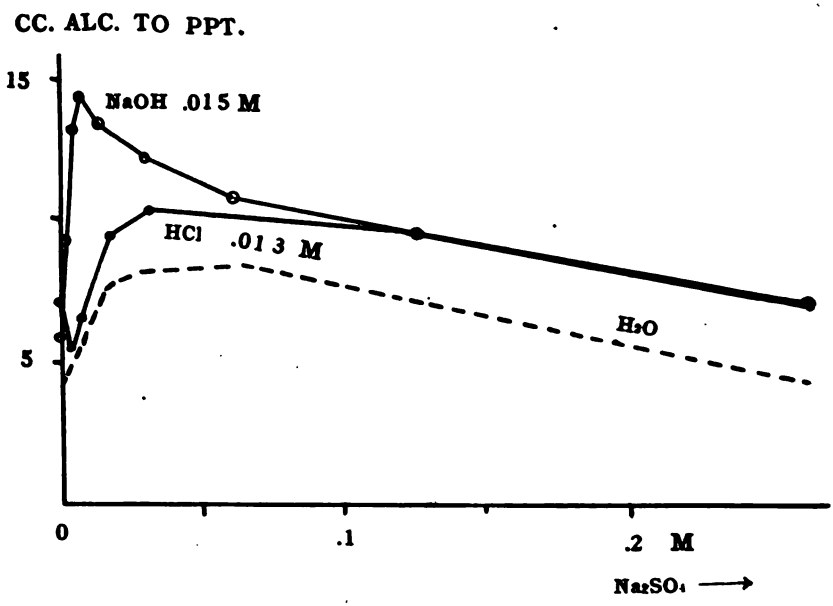


FIG. 2. Curves showing the effect of increasing concentrations of Na_2SO_4 (abscissæ) on the alcohol number of gelatin plus NaOH 0.015 M, HCl 0.013 M, and water (dotted line). The proportions of SO_4 to H at the minimum of the HCl curve are 100: 30. Na_2SO_4 is antagonistic to HCl but not antagonistic to NaOH in small concentrations. (See Table II.)

TABLE II.

Effect of Na_2SO_4 on the Alcohol Number of Gelatin plus HCl and NaOH.

Concentration of Na_2SO_4.	Cc. alcohol to precipitate in gelatin plus		
	H_2O	HCl, 0.013 M	NaOH, 0.015 M
M			
0.5	3.1	4.9	4.5
0.25	4.6	7.25	7.1
0.125	7.1	9.6	9.6
0.0625	8.3	10.2	10.8
0.0312	8.2	10.4	12.2
0.0156	7.7	9.3	13.3
0.0078	5.8	6.5	14.5
0.0039	5.1	5.5	13.2
0.00195	4.3	5.9	9.2
0.00097	4.1	6.5	7.75
0.00048	3.95	6.9	7.1
0.0	3.9	7.2	6.4

3 per cent gelatin C which stood over night at 26°C. before adding alcohol. Gelatin C is not so pure as gelatin B. Other details as in Table I. (See Fig. 2.)

Digitized by Google

NaOH and Na_2SO_4 are not antagonistic. Their relations in higher concentrations are like those for Na_3 citrate and NaOH in Fig. 3.

To determine the effects of higher concentrations of Na_3 citrate and NaOH on gelatin, the effect of increasing concentrations of Na_3 citrate was determined for gelatin made up in water, and in six concentrations of NaOH. The results for four of these concentrations and for water are plotted in Fig. 3 (see Table III). The concentrations of Na_3 citrate used are plotted on the abscissæ and the concentrations of NaOH simultaneously present in the gelatin are inserted on the curves. In the concentrations of NaOH chosen, no precipitate could be produced with alcohol unless Na_3 citrate was also present in sufficient amount. The more Na_3 citrate there is present the easier it is to precipitate. This shows antagonism. At points A and B, moreover, the combined effect is less than the effect of either Na_3 citrate or NaOH alone in those concentrations. An examination of the ordinates at B shows that small amounts of NaOH increase the alcohol number of gelatin plus Na_3 citrate, but larger amounts decrease it. This is in agreement with the fact that small amounts of NaOH hinder the precipitation of gelatin by Na_2SO_4 and Na_3 citrate, while larger amounts favor it.[14] Conversely, it may be inferred from this figure, taken in conjunction with the experiments in Fig. 2, that small amounts of Na_2SO_4 or Na_3 citrate increase the alcohol number of gelatin plus NaOH, while higher concentrations decrease it.

From this experiment the conclusion may therefore be drawn that *Na_2SO_4 and NaOH are not antagonistic in weak concentrations (of both) but that they are antagonistic in higher concentrations.*

In Fig. 4 and Table IV are shown the effects of increasing concentrations of $CaCl_2$ on the alcohol number of gelatin dissolved in water, NaOH, and acetic acid. In NaOH, the curve passes through a sharp minimum. This is due in part at least, to the formation of $Ca(OH)_2$, a small amount of which precipitated. It is probable that there is also some residual antagonistic effect upon the gelatin. In their relations to alkaline gelatin $MgCl_2$ and $AlCl_3$ behave like $CaCl_2$, the curves passing through similar minima. In acid gelatin, however, $MgCl_2$ behaves more like

[14] Figures for this will appear in a subsequent paper on the precipitation of gelatin by mixtures of electrolytes.

Digitized by Google

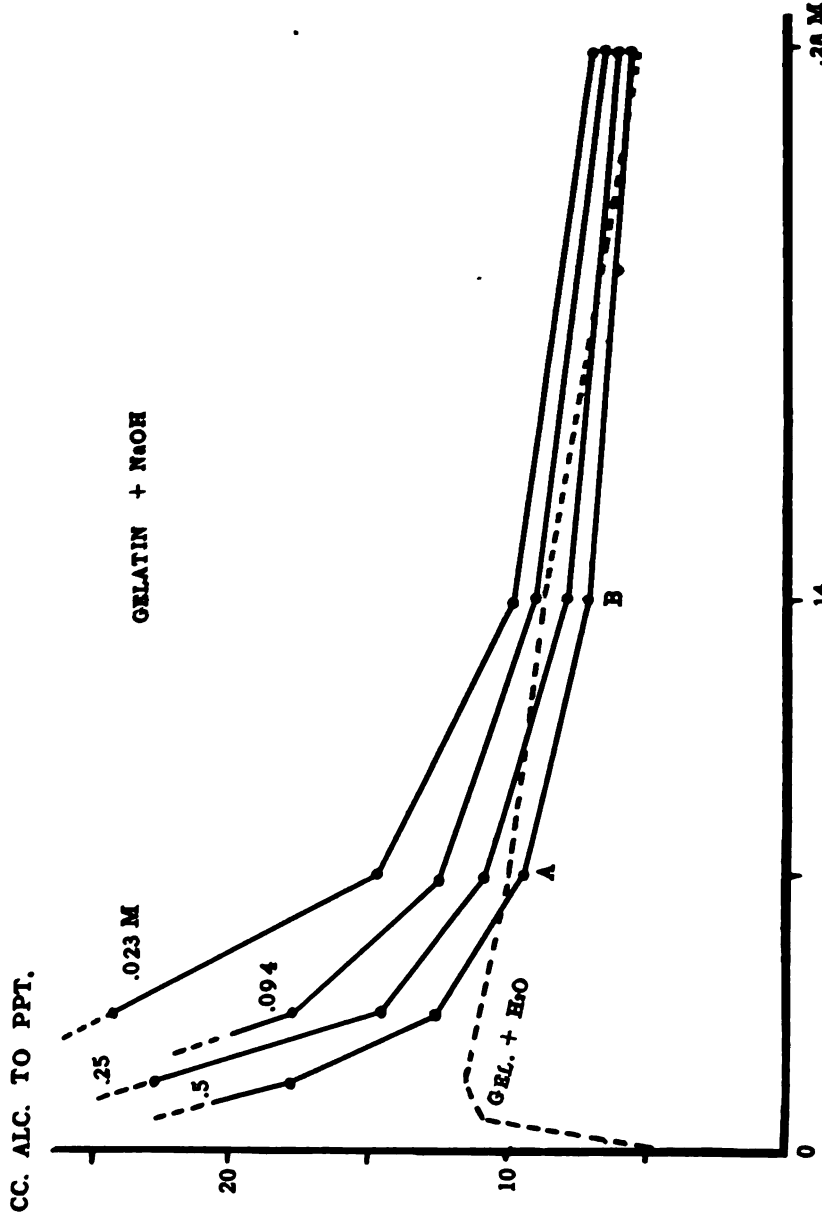


FIG. 8. Curves showing the effect of Na citrate on the alcohol number of gelatin plus NaOH of various concentrations, as noted on the curves. In pure NaOH no precipitation by alcohol is possible. (See Table III.)

Digitized by Google

NaCl because it fails to give the sharp maximum which $CaCl_2$ gives in its effect on acetic acid-gelatin, as shown in Fig. 4.

A sharp maximum like that shown for $CaCl_2$ plus acetic acid has been shown to be characteristic of the effects of acid alone on the alcohol number of gelatin. It is not found in the pure $CaCl_2$ curve as shown by the dotted line. Furthermore, an examination of these curves reveals the fact that at no point is the combined effect of $CaCl_2$ and acetic acid less than the effect of either $CaCl_2$ or acetic acid alone. $CaCl_2$ and acetic acid, at least in small concentrations, are not antagonistic in their effects.

TABLE III.

Effect of Sodium Citrate on the Alcohol Number of Gelatin plus NaOH.

Concentration of Na citrate.	Cc. alcohol to precipitate in gelatin plus NaOH.						
	0.0 M	0.0235 M	0.047 M	0.094 M	0.127 M	0.254 M	0.508 M
M							
0.28	5.4	7.0	6.5	6.5	6.6	6.1	5.6
0.14	8.8	9.7	9.6	8.9	8.9	7.9	7.1
0.07	10.0	14.7	13.7	12.4	13.6	10.7	9.4
0.035	10.9	24.4	22.0	17.8	16.5	14.6	12.6
0.0175	11.5	No ppt.	30.0+	No ppt.	22.7	22.8	17.8
0.0087	10.7	" "	No ppt.	" "	32.0+	30.0	30.0
0.0043		" "	" "	" "	No ppt.	No ppt.	No ppt.
0.0	4.5	" "	" "	" "	" "	" "	" "

2 per cent gelatin A for 15 to 20 hours at 30°C. before adding alcohol. Other details as in Table I. (See Fig. 3.)

In higher concentrations of $CaCl_2$, however, it is antagonistic to acids. Thus Fig. 5 and Table V show that acetic acid completely prevents the precipitation of gelatin by alcohol in concentrations greater than 0.1 M (see dotted line in the H_2O curve) but that when $CaCl_2$ 1.5 M is also present, precipitation is possible even in high concentrations of acetic acid. A comparison of this result with the acetic acid curves in gelatin plus sodium salts (Fig. 1) shows that although $CaCl_2$ does antagonize acids it is very much less effective in this respect than are the sodium salts, particularly those with polyvalent anions.

In low concentrations, therefore, *$CaCl_2$ is not antagonistic to acids but in higher concentrations it is.*

Digitized by Google

This experiment is of particular interest from the biological point of view, since Loeb[15] has found that the effectiveness of cations in hindering the penetration of acetic acid into the egg of *Fundulus* increases with the valence, and this result is contrary to the suggestion that antagonism is due to oppositely charged ions. While this result cannot be paralleled in gelatin by the alcohol method, the experiment recorded in Fig. 5 shows without a doubt that antagonism between acids and salts with biva-

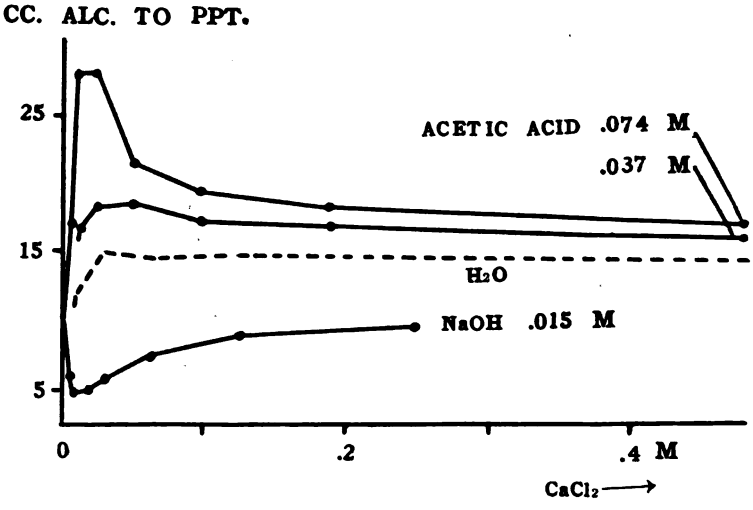


FIG. 4. Curves showing the effect of $CaCl_2$ on the alcohol number of gelatin plus NaOH and acetic acid. $CaCl_2$ is antagonistic to NaOH but not antagonistic to acetic acid. (See Table IV.)

lent cations is possible in gelatin. The writer[16] has also found that $CaCl_2$ 1.5 M will antagonize HNO_3 and $AlCl_3$ in the same way.

Fischer[15] has found, in studying the swelling of gelatin in acids, that the effectiveness of cations in hindering this swelling increases with the valence of the cation. Thus he found that $FeCl_3$ hinders swelling in HCl more than $CaCl_2$. This is in accord with Loeb's results but is the reverse of the evidence so far obtainable by the alcohol method, where no indication of antagonism between HCl and $AlCl_3$ has as yet been found. As it is impossible to follow the effects of HCl and $AlCl_3$ by this method,

[15] Loeb, *J. Biol. Chem.*, 1916, xxvii, 365.

[16] Fischer, Œdema and Nephritis, New York, 2nd edition, 1915.

Digitized by Google

TABLE IV.

Effect of $CaCl_2$ on the Precipitation by Alcohol of Acid and Alkaline Gelatin.

In acetic acid.*			In NaOH.†	
Concentration of $CaCl_2$.	Cc. alcohol to precipitate in gelatin plus		Concentration of $CaCl_2$.	Cc. alcohol to precipitate in gelatin plus NaOH, 0.015 M
	Acetic acid 0.0371 M	Acetic acid 0.0743 M		
M			M	
1.5	15.6	16.4	0.25	9.9
0.75	16.0	17.0	0.125	9.1
0.375	16.7	17.8	0.0625	7.8
0.188	17.2	18.5	0.0312	6.2
0.094	17.5	19.5	0.0156	5.2
0.047	18.8	21.5	0.0078	5.1
0.024	18.6	27.7	0.0039	6.3
0.012	16.9	27.7	0.00195	7.6
0.006	13.8	17.1	0.00097	8.4
0.003	11.4	14.3	0.00048	9.1
0.0	8.65	10.6	0.0	11.0

* 2 per cent gelatin A for 24 hours before adding alcohol.
† 3 per cent gelatin B for 15 hours at 26°C.

The NaOH and the acetic acid curves are not strictly comparable but as they are so different there is no confusion in plotting them together. Gelatin B was not so pure a grade of commercial gelatin as gelatin A. Data for the effect of $CaCl_2$ on pure gelatin are given in Table II of the first paper of this series and are plotted with the data of this table in Fig. 4. Other details as in Table I.

except in very low concentrations, it is possible that some antagonism exists. $AlCl_3$ is certainly less effective in this respect, however, than $CaCl_2$.

In this connection, reference should be made to the conclusions of Chick and Martin[17] concerning the effects of acids and alkalies on salts in albumin solutions. They found that small amounts of NaOH and HCl hindered the precipitation of albumin by Na_2SO_4 and $CaCl_2$ respectively, but larger amounts favored it.

[17] Chick and Martin, *Biochem. J.*, 1913, vii, 380.

Digitized by Google

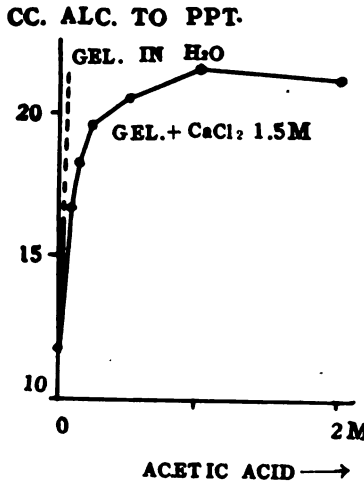


FIG. 5.

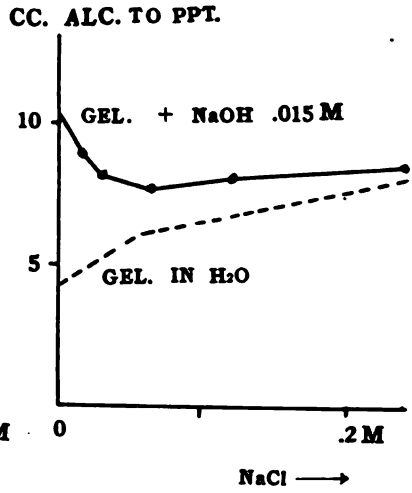


FIG. 6.

FIG. 5. Curves showing how strong concentrations of $CaCl_2$ (1.5 M) make precipitation of the gelatin by alcohol possible even in high concentrations of acetic acid. This shows antagonism. (See Table V.)

FIG. 6. Curves showing the effect of NaCl on H_2O-gelatin (dotted line) and NaOH-gelatin. Antagonism is shown by the drop of the NaOH curve. (See Table VI.)

TABLE V.

Antagonism between High Concentrations of $CaCl_2$ and Acetic Acid.

Acetic acid.*		Acetic acid in gelatin plus $CaCl_2$ 1.5 M†	
Concentration of acetic acid.	Cc. alcohol to precipitate.	Concentration of acetic acid.	Cc. alcohol to precipitate.
M		M	
0.13	No ppt.	4.17	19.3
0.065	11.6	2.08	21.4
0.033	9.25	1.04	21.7
0.016	7.75	0.52	20.7
0.008	6.85	0.26	19.8
0.0	4.95	0.13	18.2
		0.065	16.7
		0.0325	15.7
		0.0162	15.1
		0.008	14.1
		0.004	13.7
		0.0	13.2

* 2 per cent gelatin A over night at 31°C.

† 2 per cent gelatin A. Temperature regulator broke and the temperature varied; therefore it was not strictly comparable. Other details as in Table I. (See Fig. 5.)

Digitized by Google

The effect of NaCl on gelatin dissolved in NaOH is shown in Fig. 6 and Table VI. The effect of NaCl on pure gelatin is a gradual increase in the alcohol number, as shown by the dotted line. Its effect on gelatin made up in NaOH, 0.015 M, is to decrease the alcohol number until a minimum is reached, after

TABLE VI.

Antagonism between NaCl and NaOH in Their Effects on Gelatin.

Concentration of NaCl.	Cc. alcohol to precipitate in gelatin plus	
	H_2O	NaOH, 0.015 M
M		
0.25	8.0	8.45
0.125	6.85	8.05
0.0625	6.15	7.8
0.0312		8.1
0.0156		9.0
0.0	4.4	10.4

3 per cent gelatin B. Stood over night at 26°C. before adding alcohol. Other details as in Table I. (See Fig. 6.)

which the alcohol number increases gradually as in the pure NaCl curve. This shows antagonism because the combined effect of NaOH and NaCl is less than the effect of one of the electrolytes (NaOH) alone.

SUMMARY.

1. Monovalent salts (like NaCl) are antagonistic to both acids and alkalies in all concentrations.

2. Salts with bivalent cations (like $CaCl_2$) are antagonistic to alkalies but not antagonistic to acids (except in high concentrations).

3. Salts with bivalent anions (like Na_2SO_4) are antagonistic to acids but not antagonistic to alkalies except in high concentrations when the greater the concentration of either alkali or salt, the less the alcohol necessary for precipitation.

Digitized by Google

Digitized by Google

THE EFFECT OF POTASSIUM BROMATE UPON ENZYME ACTION.

By I. S. FALK and C.-E. A. WINSLOW.

(From the Department of Public Health, Yale School of Medicine, New Haven.)

(Received for publication, November 14, 1917.)

One of the most recent important advances in the practical art of breadmaking has been the introduction into the dough of a mixture of mineral nutrients known as Arkady Yeast Food.[1] This material, which was devised in the laboratories of the Mellon Institute of Pittsburg, includes salts of calcium and ammonium and a minute amount (1 part in 200,000) of potassium bromate.

The functions of calcium and ammonium as yeast foods are fairly well understood; but the reasons for the favorable action of the bromate remained somewhat obscure. It seemed possible that this salt might exert a specific stimulating action upon the proteolytic enzymes active in the fermentation. It had been shown by Chittenden and his associates[2] that bromides in certain dilutions had a marked accelerating effect on the action of ptyalin, pepsin, and pancreatin. Wohlgemuth[3] noted the same effect on ptyalin and pancreatin. More recently Thomas[4] reports a strongly stimulating influence of bromides upon amylase; and Robertson[5] in an exhaustive study of the action of trypsin on casein notes a stimulating action of potassium bromide. On the other hand, Senter,[6] and Battelli and Stern,[7] and Jappelli[8] found

[1] Allen, R. M., *Baker's Weekly*, 1916, xviii, 31.

[2] Chittenden, R. H., and Painter, H. M., *Tr. Conn. Acad. Arts and Sc.*, 1885–88, vii, 60. Chittenden, R. H., and Allen, S. E., *ibid.*, 84. Chittenden, R. H., and Cummins, G. W., *ibid.*, 108.

[3] Wohlgemuth, J., *Biochem. Z.*, 1908, ix, 10.

[4] Thomas, A. M., *J. Am. Chem. Soc.*, 1917, xxxix, 1501.

[5] Robertson, T. B., *J. Biol. Chem.*, 1906–07, ii, 317.

[6] Senter, G., *Z. physikal. Chem.*, 1903, xliv, 257.

[7] Battelli. F., and Stern, L., *Arch. internat. Physiol.*, 1907, iv, 465.

[8] Jappelli, A., *Atti XXII cong. med. int. Roma*, 1913, 405.

Digitized by Google

that oxidizing enzymes were inhibited by bromides. All these investigations were made with rather strong concentrations of salts; and nothing, so far as we are aware, has been published on the question of the action of bromates upon the enzymes, trypsin and pancreatin. An investigation of this point was therefore undertaken at the request of the Ward Baking Company of New York.

Effects on Trypsin.

The technique of the trypsin experiments was as follows: A buffer solution was prepared, made up of 40 cc. of 0.067 M K_2HPO_4, 10 cc. of 0.067 M KH_2PO_4, and 50 cc. of water; pH value 7.1. Definite amounts of casein and trypsin were added as stated in the protocol of the individual experiments, and to a series of bottles of the resulting solution were added varying amounts of potassium bromate. The bottles were then incubated at 37°C. for the periods stated in the protocols.

At the end of the incubation period the amino-acid content of each bottle was determined by the following method, and the excess produced by the action of the trypsin found by subtracting the amino-acid content of a control containing casein alone. Each bromate dilution was tested in triplicate.

Method of Titration.

1. Add phenolphthalein to incubated solution.
2. Add $Ba(OH)_2$ (saturated solution) to deep red color—removal of phosphates.
3. Pour into graduated cylinder.
4. Rinse incubation bottle with small quantity of distilled H_2O. Pour rinsings into cylinder.
5. Make up to 100 cc. with distilled H_2O.
6. Filter through paper into bottle.
7. Measure off 50 cc. of filtrate and pour into cylinder.
8. Neutralize to faint pink with 0.2 N HCl.
9. Add 0.2 N NaOH to desired titration color.
10. Add 15 cc. of neutralized HCHO.
11. Titrate with 0.2 N NaOH to same color as in (9).
12. Express results in terms of cc. of 0.2 N NaOH to neutralize after addition of neutralized HCHO.

In a series experiment, preserve control by addition of $CHCl_3$ and keeping in a dark place.

Digitized by Google

Experiment I.—June 11, 1917. 2 gm. of casein, 0.4 gm. of Merck's trypsin, per 100 cc. of solution. Digestion period, 4 hours.

Solution.	Initial pH.	Final pH.	Titrations of 50 cc. portions.*					
			Initial amino-acids. Cc. 0.2 N NaOH to neutralize.	Final amino-acids. Cc. 0.2 N NaOH to neutralize.		Increase in amino-acids.	Titratable acid. Final cc. 0.2 N NaOH.	
Buffer + casein	6.1	5.9	1.80	1.85		0.05	7.5	
" " + trypsin	6.1	5.9	1.80	7.15 6.75 6.05	6.65	4.85	11.60 11.45 12.00	11.70
" " " + $KBrO_3$ 1 : 10,000	6.1	5.9	1.80	6.85 6.45 6.30	6.55	4.70	11.80 12.30 12.15	12.10
" " " " 1 : 50,000	6.1	5.9	1.80	7.00 6.65 7.20	6.95	5.15	12.45 12.00 11.95	12.15
" " " " 1 : 100,000	6.1	5.9	1.80	8.60 6.95 8.20	7.90	6.10	10.70 11.05 10.90	10.90

* Results expressed to nearest 0.05.

Digitized by Google

The results of check determinations in this experiment were a little irregular, but the tubes containing 1 part of bromate in 100,000 showed a significant increase in digestion.

Experiment II.—June 16, 1917. 2 gm. of casein, 0.4 gm. of Merck's trypsin, per 100 cc. of solution. Digestion period, 4½ hours.

Solution.	Titration of 50 cc. Increase in amino-acids during digestion. Cc. 0.2 N NaOH to neutralise.	
Buffer + cascin + trypsin.	5.2 5.9 6.0	5.7
" " " + $KBrO_3$ 1: 10,000	6.2 6.4 5.8	6.1
" " " " 1: 50,000	5.7 5.9 6.0	5.9
" " " " 1: 100,000	6.6 6.2 6.0	6.3
" " " " 1: 200,000	6.4 6.7 7.2	6.8
" " " " 1: 300,000	6.2 6.2 5.8	6.1
" " " " 1: 500,000	5.9 6.0 5.0	5.6

The results of this experiment again show considerable individual variations, but with higher amino-acid contents at bromate concentrations of 1 in 100,000 and 1 in 200,000, the latter being most marked.

Digitized by Google

Experiment III.—June 29, 1917. 2 gm. of Merck's casein, 0.1 gm. of Fairchild Brothers and Foster's trypsin, per 100 cc. Digestion period, 3½ hours.

Solution.	Titration of 50 cc. Increase in amino-acids during digestion. Cc. 0.2 N NaOH to neutralize.
Buffer + casein + trypsin.	2.4 2.0 2.1 2.0
" " " + $KBrO_3$ 1: 10,000	2.7 2.7 2.7 2.7
" " " " 1: 50,000	3.3 3.3 3.3 3.3
" " " " 1: 100,000	3.6 3.1 3.4 3.6
" " " " 1: 150,000	4.0 5.3 4.5 4.1
" " " " 1: 200,000	2.6 4.2 3.4 3.5
" " " " 1: 500,000	3.0 2.7 2.7 2.5

In this experiment the digestion increases regularly with decreasing amounts of bromate to a concentration of 1 part in 150,000 and then decreases again with further dilution of the salt. Here as in other experiments it appears, however, that even certain high concentrations of bromate (1 part in 10,000) are rather favorable than otherwise to the process of enzyme action.

Digitized by Google

Experiment IV.—July 13, 1917. 2 gm. of Merck's casein, 0.2 gm. of Fairchild Brothers and Foster's trypsin, per 100 cc. Digestion period, 3 hours.

Solution.	Titration of 50 cc. Increase in amino-acids during digestion. Cc. 0.2 N NaOH to neutralise.	
Buffer + casein + trypsin.	2.7 2.85 2.7	2.75
" " " + $KBrO_3$ 1:100,000	2.8 3.0 3.1	3.0
" " " " 1:120,000	3.25 3.1 4.4	3.6
" " " " 1:140,000	3.85 3.3 3.6	3.6
" " " " 1:160,000	4.1 4.25 4.1	4.15
" " " " 1:180,000	4.25 4.0 4.2	4.15
" " " " 1:200,000	5.5 5.8 5.8	5.7
" " " " 1:250,000	4.9 4.7 4.0	4.5

Again the result is an increase of enzyme action under the influence of bromate up to a concentration of 1 part in 200,000. In both these last experiments the digestive action was more than doubled by the stimulating effect of the bromate.

Digitized by Google

Effects on Pancreatin.

The influence of bromate upon the action of pancreatin was next studied in the same way. The buffer solution in this case was made up with 50 cc. of 0.067 M K_2HPO_4, 17.5 cc. of 0.2 N NaOH, and 32.5 cc. of water, giving an initial pH value of 10.8. The titration method was the same as outlined above.

Experiment V.—July 30, 1917. 3 gm. of Merck's casein, 0.5 gm. of Merck's pancreatin, to 100 cc. Digestion period, 5½ hours.

Solution.					Increase in amino-acids in 25 cc. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein.						
"	"					0.85
"	"					
"	"	+ pancreatin.			4.00	
"	"	"			4.20	3.95
"	"	"			3.70	
"	"	"	+ $KBrO_3$	1:100	3.90	
"	"	"	"		3.45	3.95
"	"	"	"		4.45	
"	"	"	"	1:500	3.85	
"	"	"	"		3.60	3.60
"	"	"	"		3.40	
"	"	"	"	1:1,000	2.70	
"	"	"	"		3.10	3.00
"	"	"	"		3.10	
"	"	"	"	1:10,000	3.70	
"	"	"	"		3.35	3.50
"	"	"	"		3.50	

This experiment appears to indicate that bromate in strong concentrations (1:100 to 1:10,000) exerts an inhibitive action upon pancreatin.

Digitized by Google

Experiment VI.—July 30, 1917. 3 gm. of Merck's casein, 0.5 gm. Merck's pancreatin, to 100 cc. Digestion period 4 hours.

Solution.	Increase in amino-acids in 25 cc. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein.	0.80	
" "		
" "		
" " + pancreatin.	2.95	
" " "	2.75	2.90
" " "	2.95	
" " " + $KBrO_3$ 1:200	2.85	
" " " "	2.85	2.80
" " " "	2.80	
" " " " 1:400	2.40	
" " " "	2.60	2.50
" " " "	2.55	
" " " " 1:600	2.10	
" " " "	2.35	2.25
" " " "	2.30	
" " " " 1:50,000	2.60	
" " " "	2.35	2.45
" " " "	2.40	
" " " " 1:100,000	2.50	
" " " "	2.65	2.60
" " " "	2.60	
" " " " 1:150,000	3.00	
" " " "	3.15	2.95
" " " "	2.75	
" " " " 1:200,000	3.45	
" " " "	3.50	3.70
" " " "	4.10	
" " " " 1:250,000	3.30	
" " " "	3.75	3.55
" " " "	3.55	

Again the stronger bromate solutions (1:200 to 1:100,000) appear to inhibit the action of the enzyme, while higher dilutions (1:200,000 and 1:250,000) show a distinct increase in digestion products.

Digitized by Google

Experiment VII.—Aug. 10, 1917. 2 gm. of Merck's casein, 0.5 gm. of Merck's pancreatin to 100 cc. In this experiment large portions of the solution (95 cc.) were put up in bottles and incubated for 24 hours, bacterial action being held in check by 5 cc. of toluene on the surface of the liquid in each bottle. The bottles were shaken every 10 minutes for the first 4 hours.

Solution.	Increase in amino-acids in 25 cc. Cc. 0.2 N NaOH to neutralize.	
Buffer + casein.	0.90	
" "	0.90	0.90
" "	0.90	
" " + pancreatin.	4.75	
" " "	4.70	4.70
" " "	4.70	
" " " + $KBrO_3$ 1:100	4.60	
" " " "	4.50	4.50
" " " "	4.40	
" " " " 1:500	4.30	
" " " "	4.45	4.35
" " " "	4.35	
" " " " 1:1,000	4.20	
" " " "	4.45	4.35
" " " "	4.35	
" " " " 1:10,000	4.70	
" " "	4.60	4.70
" " "	4.85	
" " " " 1:50,000	4.90	
" " " "	4.90	4.90
" " " "	4.85	
" " " " 1:100,000	5.30	
" " " "	5.35	5.20
" " " "	4.90	
" " " " 1:250,000	4.90	
" " " "	5.25	5.10
" " " "	5.10	
" " " " 1:500,000	4.85	
" " " "	4.85	4.85
" " " "	4.90	
" " " " 1:1,000,000	4.60	
" " " "	4.50	4.60
" " " "	4.75	

Digitized by Google

The same results are again apparent, slight inhibition by concentrations of 1: 100 to 1: 1,000, and slight stimulation by concentrations of 1: 50,000 to 1: 500,000.

CONCLUSIONS.

1. Potassium bromate appears to exert consistently favorable influence upon the digestion of casein by trypsin *in vitro* in the dilutions studied, the action being most marked at bromate concentrations of 1 part in 100,000 to 1 part in 200,000.

2. Potassium bromate in concentrations of 1 part or more in 10,000 appears to exert a slight inhibitive influence upon the digestion of casein by pancreatin, while in higher dilutions (1 part in 200,000 or 1 part in 250,000) it appears to exert a stimulating action.

Digitized by Google

CELL PENETRATION BY ACIDS.

IV. NOTE ON THE PENETRATION OF PHOSPHORIC ACID.

By W. J. CROZIER.

Contributions from the Bermuda Biological Station for Research, No. 83.

(Received for publication, December 12, 1917.)

I. Measurements of the relative speeds with which acids penetrate protoplasm, as carried out by different observers upon widely different tissues containing natural indicators, have shown in general a noteworthy agreement with regard to the way in which the various acids are arranged in order of their penetrating ability (Harvey, 1914; Crozier, 1916, *a*, *b*; Haas, 1916, *a*). This must indicate some fundamental fact about the organization of protoplasm, since some of the relations observed are such as might not be predicted. It is, then, of interest to examine an instance in which minor disagreement has been found. The agreement referred to obtains in acid solutions which are 0.01 N by titration, and it has been shown (Crozier, 1916, *a*, *b*) that, owing to the several forms of the penetration curves, the sequence of increasing penetrating ability may vary with the concentration at which the comparisons are made; and it was pointed out that a similar condition obtained with regard to comparisons at any one hydrogen ion concentration. Any special influence affecting the determination of the change of indicator by a particular acid may cause the form of its penetration curve to be different with different tissues, according to the variation of this special factor. Thus, among other possibilities, variations in the composition of the surface layer of the cell, or in the "buffer" conditions under which the intracellular titration takes place, or variations in the behavior of the different indicators used, might be appealed to to explain observed differences in the relative order of penetration for different tissues; and it might be expected that an acid whose salts have special significance in connection with protoplasm would be one to show characteristic variations from cell to cell.

Now, Haas (1916, *a*) found that for one kind of plant material used in his experiments, the petals of *Browallia*, phosphoric acid produced the color change signifying penetration more rapidly, at 0.01 N H^+ concentration, than did oxalic, tartaric, lactic, citric, or acetic acids; whereas, with the perianth cells of a hyacinth, this took place more slowly than in oxalic acid. Harvey (1914) observed the penetration of phosphoric acid, at this concentration, to occur with about the same speed as malonic or tartaric, but more slowly than with lactic or oxalic.

Digitized by Google

For the utilization of the results of these penetration studies it was necessary to know something about the action of this acid upon the surface of cells; so experiments were made to determine its behavior with the tissue of *Chromodoris zebra;* the results throw some light upon the factors which may cause the apparent speed of penetration by different acids, as observed by this method, to vary in different tissues.

II. The technique in these measurements of the penetration of *o*-phosphoric acid was essentially as has previously been explained (Crozier, 1916, *a*, *b*). Some observations were repeated for certain acids giving penetration curves lying near that of phosphoric; these experiments sufficed to show that the curves previously published could be relied upon for comparison. Two series of experiments were made with phosphoric acid, at the same time but at different temperatures. The average penetration times obtained in the two series, as shown in Table I, differ to

TABLE I.

The apparent time required for the penetration of mantle tissue of *Chromodoris* by H_3PO_4 at different concentrations and at two temperatures; penetration time in minutes; figures in brackets obtained only in favorable cases (about 75 per cent).

Temperature.	Concentration, normal.							
	1.34	0.67	0.134	0.067	0.013	0.010	0.007	0.005
°C.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*		
24.8	2.5	4.2	5.5	7.1	14.3	[20]	∝	∝
27.0	2.2	3.7	4.9	6.0	13.0	[18.0]	∝	∝

a degree which will serve to illustrate the delicacy of the method (Fig. 1). Observations were made at 27.0° and at 24.8°; the temperature of the sea water from which the chromodorids had been recently obtained was 24–25°.

III. The average apparent penetration times of phosphoric acid, from different concentrations, are plotted in Fig. 2. It is seen that from low concentrations the penetration appears abnormally slow, in comparison with some other acids; or that the increase in speed of penetration with higher concentrations is unusually rapid. From Fig. 2 it will be evident that at any particular concentration (*e.g.*, 0.01 N) the apparent relative position of phosphoric acid in the series will depend upon the intensity of

Digitized by Google

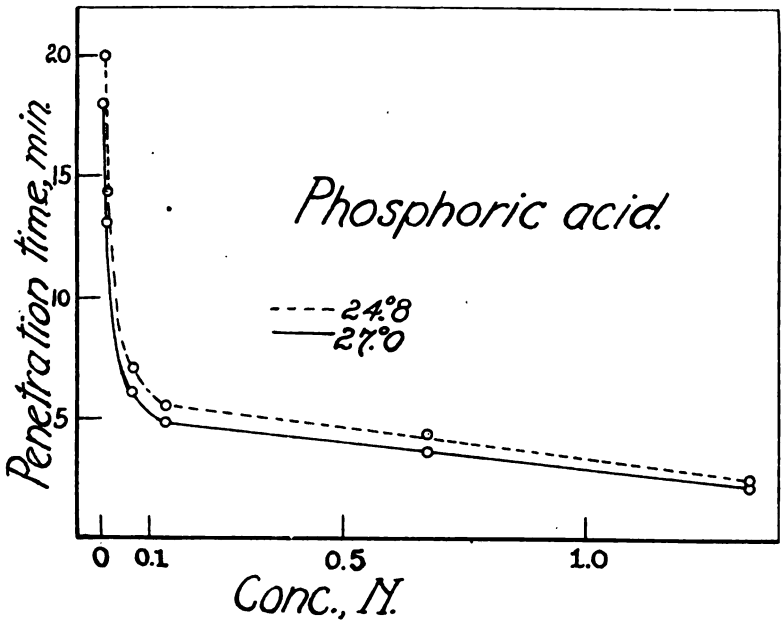


FIG. 1. The apparent penetration of phosphoric acid at two temperatures.

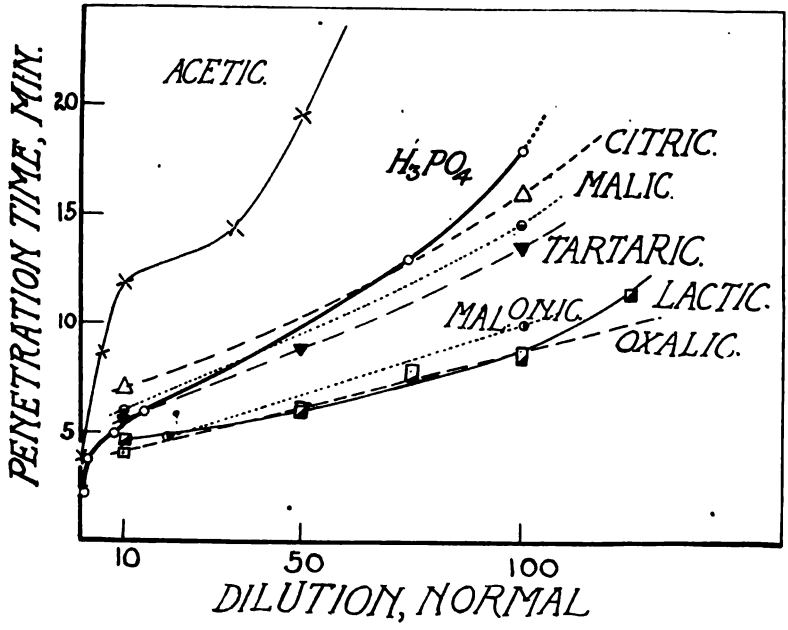


FIG. 2. The apparent penetration of H_3PO_4 compared with that of other acids (27.0°).

Digitized by Google

the factor responsible for the rapidly increasing slope of its penetration curve. This factor is probably concerned, in part, with differences in the kind and quantity of intracellular materials with which the phosphoric acid may react to produce "buffer" substances. Thus in four tissues which are available for comparison, the penetration relations of H_3PO_4 appear as follows (at 0.01 N).

TABLE II.

Source of tissue.	Transition point of indicator.	Order of apparent penetration.
	pH	
Chromodoris.	5.6	Oxalic, lactic > malonic > tartaric > malic > citric > H_3PO_4 > acetic.
Stichopus. *	(3.0?)	Lactic, oxalic > malonic, tartaric, H_3PO_4 > malic, citric > acetic.
Browallia.†	6.0–5.0‡	H_3PO_4 > oxalic > tartaric > lactic, citric > acetic.
Hyacinth.†	4.0‡	Oxalic > H_3PO_4 > tartaric > lactic > citric, acetic.

* Harvey, 1914. † Haas, 1916, *a*. ‡ Haas, 1916, *b*.

These differences appear in further part accounted for by the greater density of the *Chromodoris* tissue. The case of H_3PO_4 is somewhat different from that of caprylic acid, whose penetration cannot be observed at all by this method because of its relatively low solubility in the aqueous phase containing the indicator, although saturated solutions of this acid will produce color change in water solutions of the pigment.

IV. The actual speed of protoplasmic penetration by H_3PO_4 is probably much greater than its apparent magnitude. This is shown by the way in which the speed of diffusion rapidly increases with increasing concentration. In solutions of 0.01 N with respect to H^+, Haas (1916, *a*) observed that this acid penetrated about as rapidly as other strong acids (HCl, HNO_3), but less rapidly than acetic, formic, or lactic acids. At 0.056 N total concentration, H_3PO_4 solutions are about 0.01 N in hydrogen ions. From the curve in Fig. 1 it is seen that at this concentration the penetration time of phosphoric acid is 6.4 minutes; under similar conditions the figures for several other acids are: HCl, 7.6; HNO_3, 8.4; formic < 1.0; lactic, < 2.0. The inward diffusion of H_3PO_4,

Digitized by Google

at higher concentrations, appears, then, to be somewhat more rapid than its ionization would demand.

The figures in Table I show that at dilutions above 0.01 N the penetration of phosphoric acid is not detectable with the tissue used. It requires an external concentration of 0.01 N acid to affect color transition. In favorable cases it is found that this color transition occurs in about 18 minutes. If this limiting concentration is subtracted from all higher concentrations studied, a truer idea is obtainable of the real penetrating ability of the acid. It is necessary also to subtract from the reciprocal of each observed penetration time the reciprocal of that for the limiting quantity of acid. This reduction is carried out in Table III. The same procedure has been used with data previously obtained for other acids, and leads to some interesting conclusions; it is believed to yield information concerning the primary action of acids upon the surfaces or outer layers of cells, and in the case of strong acids it can be shown that the peculiarities of their relations depend upon reactions with proteins.

TABLE III.

The corrected concentrations and speeds of penetration for H_3PO_4 (27°). Concentration, N; speed, $\frac{1}{\text{min.}} \times 10^3$ (27°).

Concentration.		$\frac{1{,}000}{P.\ T.'}$
Nominal.	Corrected.	
1.34	1.33	398.5
0.67	0.66	215.5
0.134	0.124	148.5
0.067	0.057	111.5
0.0134	0.0034	20.4
0.010	—	—
0.007	—	—

Fig. 3 exhibits the penetration relations of phosphoric acid as shown in the corrected data (Table III); measurements with HCl are included for comparison. The significant feature of these curves lies in the fact that they are composed of two straight lines, representing the respective predominance of two different proc-

Digitized by Google

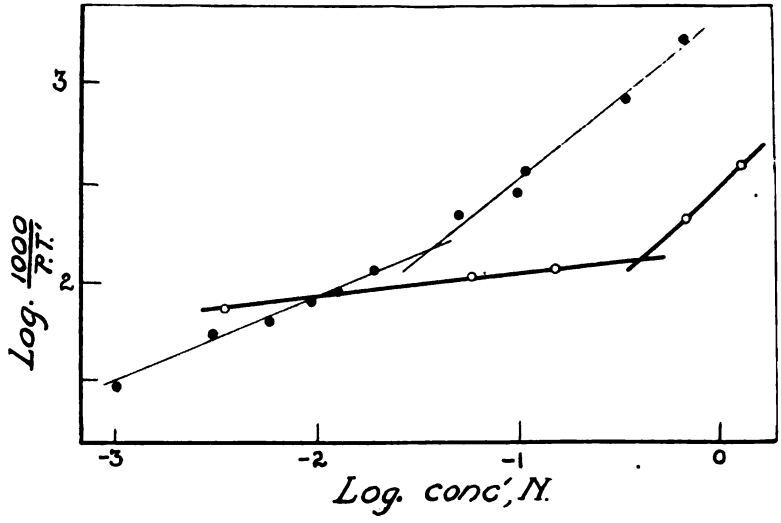


FIG. 3. The relation between speed of tissue penetration and acid concentration in solutions of HCl (light line) and H_3PO_4 (heavy line), when the formol concentration and apparent time of penetration have been corrected for the quantity of acid necessary to induce color change in the indicator system studied (27.0°).

esses. This general result is also obtained with other acids. Each part of these plots follows an equation of the form

$$\log\left(\frac{1}{P.\ T.'}\right) = \frac{1}{n}\log\ (\text{concentration}') - \log k$$

(*P. T.*, observed penetration time, *P. T.'*, corrected penetration time, k a constant), which is similar to the Freundlich formula for the adsorption isotherm and may in fact be derived from this formula by the aid of assumptions such as are commonly made in applying it. The curves shown in Fig. 3 have, however, no probable reference to adsorption; what they do express is the relation between the concentration of acid external to the tissue and its ability to force through the tissue a quantity of acid sufficient to induce the intracellular color change. With H_3PO_4 this ability increases very slowly up to a point where the osmotic pressure of the acid is adequate rapidly to overcome the resistance to its diffusion, which is produced in the tissue by the acid itself; beyond

Digitized by Google

this point the inward diffusion of acid is very rapid, and the time for the observed effect is nearly proportional to the dilution of the acid. It may be stated here that the temperature coefficient of penetration speed, for this latter portion of the curves, is of the order $Q_{10} = 2.0$, indicating the chemical nature of the principal factor governing the penetration (as might be expected). The coagulative action of H_3PO_4 is so great that the resistance to subsequent diffusion is overcome only very slowly until a high concentration of the acid is used. From these facts it may be inferred that the primary action of the H_3PO_4 is more intense than that, *e.g.*, of HCl (at equal concentration). If comparison is made at a point in the reduced data (Fig. 3) where the $C_H = 0.01$ N, it will be noticed that the H_3PO_4 is slightly more active than HCl. These considerations explain why the apparent speed of penetration of H_3PO_4 from higher concentrations gives a more correct idea of the primary effect of this acid upon the tissue used; this is not the case with some other acids.

SUMMARY.

V. The relative apparent speed with which H_3PO_4 penetrates different tissues is conspicuously modified by the density of the cells concerned, and by the proportion of "buffer" substances present. When the gross observations of apparent penetration time are corrected by consideration of the limiting concentration of acid inducing visible color change, it is found that the observations for each acid fall upon two intersecting curves of the form:

$$\frac{1}{P.T.'} = k\ (\text{concentration}')^{\frac{1}{n}}$$

which does not, however, have reference to adsorption. With mantle tissue of *Chromodoris* the coagulative action of H_3PO_4 is so pronounced that a relatively high concentration must be used before there is marked acceleration in the speed with which the quantity of acid sufficient to react with the indicator is forced to diffuse into the tissue. The primary (surface) action of H_3PO_4 is greater than is made evident by its apparent penetrating power, and is also (probably) somewhat greater than its hydrogen ionization would demand.

AGAR'S ISLAND, BERMUDA.

Digitized by Google

REFERENCES.

Crozier, W. J., 1916, *a*, Cell penetration by acids, *J. Biol. Chem.*, xxiv, 255–279.

Crozier, 1916, *b*, Cell penetration by acids, III, *J. Biol. Chem.*, xxvi, 225–230.

Dale, D., and Mines, G. R., 1911, The action of acids on skeletal muscle, *J. Physiol.*, xlii, pp. xxix–xxx.

Haas, A. R., 1916, *a*, The permeability of living cells to acids and alkalies, *J. Biol. Chem.*, xxvii, 225–232.

Haas, 1916, *b*, The acidity of plant cells as shown by natural indicators, *J. Biol. Chem.*, xxvi, 233–241.

Harvey, E. N., 1914, Cell permeability for acids, *Internat. Z. physik-chem. Biol.*, i, 463–478.

Digitized by Google

STUDIES OF EXPERIMENTAL SCURVY.

II. THE INFLUENCE OF GRAINS, OTHER THAN OATS, AND SPECIFIC CARBOHYDRATES ON THE DEVELOPMENT OF SCURVY.*

By W. PITZ.

(From the Department of Agricultural Chemistry of the University of Wisconsin, Madison.)

(Received for publication, January 29, 1918.)

In a recent publication from this laboratory McCollum and Pitz (1) put forth the theory that scurvy in the guinea pig is not the result of a deficiency of any specific protective substance, but that it is the result of the absorption of toxic substances arising from putrefaction in the cecum due to an undue retention of feces. In the present paper further proof is given in substantiation of this theory.

Hull and Rettger (2) showed with white rats and also with men fed on a high protein diet that the bacterial flora of the intestine could be changed from a putrefactive flora to an acidophilus flora by the ingestion of lactose in the diet. Other carbohydrates as sucrose, dextrose, maltose, dextrin, levulose, and galactose, produced some change in the intestinal flora of the rat but the change was not nearly so pronounced as in the case of the lactose. When lactose was fed with a high protein diet the flora was almost completely changed to the acidophilus type with but few or no evidences of putrefactive bacteria. With the other carbohydrates used, both types of bacteria were found but the number of putrefactive organisms was decreased and the number of acidophilus organisms increased as compared with the flora of an animal receiving a high protein diet, without the addition of a carbohydrate.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

Digitized by Google

If scurvy is the result of the absorption of putrefactive products arising from the putrefaction of feces in the cecum, then any substance which will improve or change the intestinal flora from a putrefactive to an acidophilus type should prevent the onset of the symptoms of scurvy. In order to test this supposition various groups of guinea pigs were fed oat meal and whole milk, plus a carbohydrate.

Chart 1 shows the remarkable recovery of a guinea pig when fed lactose after it had shown signs of very severe scurvy. The animal had swollen joints and was able to move about only with great difficulty. It was very sensitive and cried when touched. When 20 per cent of lactose was added to the oats the condition of the animal was bettered in a week. It gained in weight and improved rapidly. In 2 weeks it was able to walk about with ease and it soon became very active. The knees always remained a little swollen, but otherwise the animal appeared normal in all respects. When the animal was killed at the end of the 19th week, it showed no symptoms of scurvy except for the slight swelling of the knees. There were no hemorrhages at the knees or at the costochondral joints of the ribs.

Chart 2 demonstrates the protective action of lactose in a ration of rolled oats and milk. The animals lost weight for about 3 weeks, but at no time did they show any evidence that they were suffering from scurvy. After the 3rd week the animals gained in weight, were vigorous, very sleek in appearance, and very active. Animal 2 lost weight after the 12th week and died at the end of the 18th week. Postmortem examination failed to reveal any of the characteristic symptoms of scurvy or the cause of death. The other animals were killed at the end of the 20th week and a postmortem examination was made on each one. They were all in excellent condition, and as far as I was able to determine they were normal in all respects. The lactose not only must have changed the intestinal flora of the tract as can be assumed from the work of Rettger, but in the amounts used it acted to some degree as a laxative. Since milk was fed *ad libitum* it cannot be argued that any specific substance was added with the lactose which was not already present in considerable amounts.

Chart 3 demonstrates that although 10 per cent of lactose was beneficial to some degree it was not sufficient to protect the ani-

Digitized by Google

mals from scurvy for more than a few weeks when fed rolled oats and milk.

Charts 4, 5, 6, and 7 show the effect of feeding dextrin, dextrose, sucrose, and starch with the standard ration of rolled oats and whole milk. In every case some protection was afforded, although in the case of dextrose the protection was but slight. The appearance of these animals was better than that of animals receiving only oats and milk, but their appearance was not nearly so good as that of the animals shown in Chart 2. The growth curves show considerable improvement over the growth curves of animals receiving no additional carbohydrate (compare Charts 4, 5, 6, and 7 with Chart 8). This slight protection afforded by dextrin, dextrose, sucrose, and starch is explained by the findings of Hull and Rettger as cited above and also by the work of Hirschler (3). The latter showed that, *in vitro*, sucrose, glycerol, dextrin, starch, and lactic acid completely prevented the formation of indole, scatole, and phenol over a period of 10 days, from proteins which were inoculated with cultures of putrefactive organisms. In the animal body the same carbohydrates did not completely prevent the formation of indole, scatole, and phenol in the feces of dogs fed a high protein diet, but they did materially decrease the amounts of these products formed as compared with the amounts found in the feces of dogs fed a high meat diet but receiving no carbohydrates.

These results demonstrate beyond a doubt that a putrefactive flora in the intestine of the animal is instrumental in the production of scurvy, and that a diet which will cause a change in the flora from a putrefactive type to an acidophilus type will protect the animal from scurvy.

Production of Scurvy by Grains Other than Oats.

Lusk (4) makes the following statement, "It is important to remember that it has never been demonstrated that any kind of unmilled grain will produce scurvy." If scurvy is the result of the retention of feces, and there can be little doubt that such is the case, then any ration which will produce feces difficult to eliminate should produce scurvy. It has long been known that milk when taken in large quantities will cause severe constipa-

Digitized by Google

tion. This being true, then any grain as barley, wheat, corn (maize), etc., when fed with an abundant supply of milk should produce scurvy in the guinea pig. To test this point whole corn, wheat, and barley were ground, and the ground grains fed to guinea pigs together with all the milk they would consume.

Charts 9, 10, and 11 show the results of these experiments. It will be noted that these curves show decidedly better growth than do the curves of guinea pigs fed on whole oats and milk (see Chart 12). The animals were in better condition and did not show symptoms of scurvy as soon as did oat-fed animals. The mortality of the animals receiving corn, wheat, or barley was very high, and postmortem examination of the animals that died showed the characteristic lesions of scurvy in all but a few cases. One animal on the barley and milk ration was still alive at the end of 18 weeks. This animal shows no evidence of having scurvy at the present time. From the 4th week to the 8th week, however, it was in very poor condition and showed signs of scurvy, but after the 8th week it slowly improved and at the present time is in excellent condition. This ability of an animal that has scurvy to slowly recover without the administration of any remedial agents has been observed at various times. It occurs on different rations and is probably due to unusual vigor and resistance of the individual.

Two animals are still alive on the corn and milk ration, but although they are gaining in weight they both have swollen knee joints and are very sensitive. The coats are rough and the animals appear to be poorly nourished, in spite of the fact that they are gaining in weight. This ability of a scurvy animal to gain in weight has been noted by Jackson and Moore (5) in their work with guinea pigs.

Two animals are alive on the wheat and milk diet at the time of writing this paper. One of these, No. 2, shows no symptoms of scurvy although it has been losing weight for the past 4 weeks. The other animal, No. 4, has swollen knee joints, walks with considerable difficulty, and shows signs of severe scurvy.

The lower efficiency of rolled oats and whole oats as compared with other grains in the nutrition of the animal is due in part to the peculiar property of oats to produce very pasty feces which are difficult to eliminate. This lower efficiency of the oat

Digitized by Google

kernel, due to its formation of pasty feces, when fed rats was also noted by McCollum, Simmonds, and Pitz (6), in their investigation of the dietary deficiencies of the oat kernel.

Animals that received corn, wheat, or barley and milk were protected from scurvy by the addition of lactose to the ration. With these grains, however, 10 per cent of lactose when added to the grain was just as effective in preventing scurvy as was 20 per cent of lactose when fed with oats. 20 per cent of lactose when fed with corn, wheat, or barley caused abnormal fermentation in the tract of the animal and caused the abdomen to be greatly distended with gas.

These results show that scurvy can be produced in the guinea pig by feeding a ration of unmilled grains and milk.

I wish to thank Professor E. B. Hart for the many suggestions which he offered and especially for his suggestion of the feeding of lactose and the various grains.

BIBLIOGRAPHY.

1. McCollum, E. V., and Pitz, W., *J. Biol. Chem.*, 1917, xxxi, 229.
2. Hull, T. G., and Rettger, L. F., *Centr. Bakteriol., 1te Abt., Orig.*, 1915, lxxv, 219.
3. Hirschler, A., *Z. physiol. Chem.*, 1886, x, 306.
4. Lusk, G., Elements of the Science of Nutrition, Philadelphia, 3rd edition, 1917, 365.
5. Jackson, L., and Moore, J. J., *J. Infect. Dis.*, 1916, xix, 478.
6. McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxix, 341.

Digitized by Google

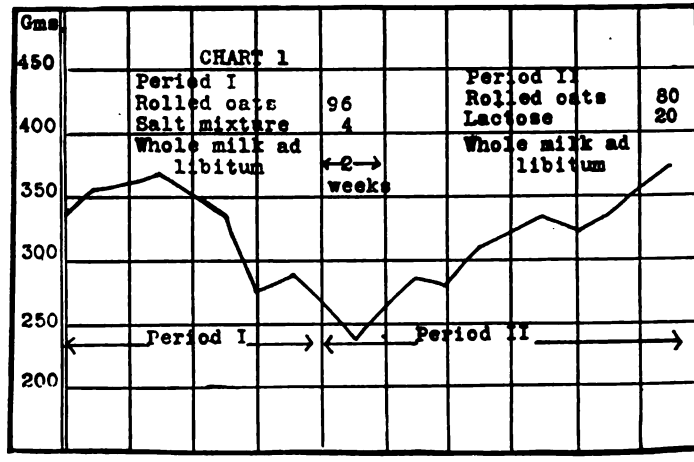


CHART 1. This chart illustrates the remarkable recovery of an animal when fed lactose in the ration. During the first period the animal received a ration consisting of oat meal and a salt mixture and whole milk. The salt mixture consisted of inorganic elements in the same proportions as found in orange juice, but acetic acid was put into the mixture in place of citric acid. It was shown in our earlier paper that this same salt mixture, but containing citric instead of acetic acid, prevented the development of scurvy in a diet of rolled oats and heated carrots. During the second period the animal was given a ration consisting of rolled oats 80 and lactose 20, plus whole milk. At the beginning of the lactose feeding the animal was in a most miserable condition. It was very weak, moved about with great difficulty, and was very sensitive. After the lactose was introduced into the ration the animal gained in weight rapidly and showed marked improvement. All symptoms of scurvy disappeared except for a slight swelling at the knee joints. Postmortem examination failed to reveal the characteristic lesions of scurvy.

Composition of salt mixture.

	gm.
Ca citrate	2.261
$MgCl_2 6H_2O$	1.270
K citrate	2.578
NaCl	0.787
K_2HPO_4	0.837
Acetic acid	0.960
Sucrose	8.270

Digitized by Google

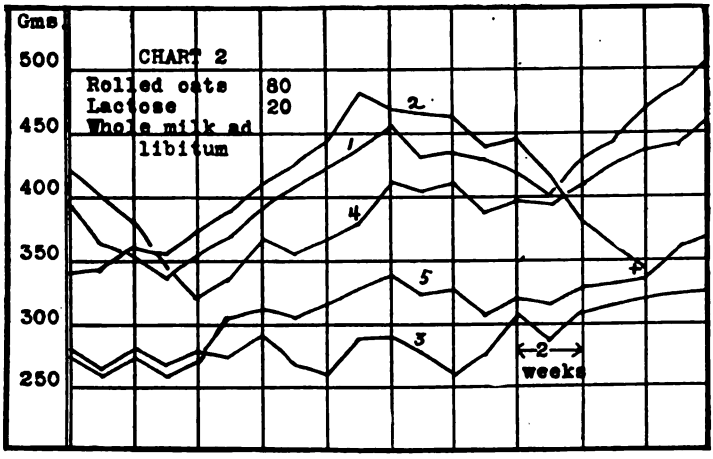


CHART 2. This record gives further evidence of the beneficial effects resulting from the ingestion of 20 per cent of lactose with an oat and milk diet (compare with Chart 8). All the animals except No. 2 were in excellent condition throughout the experiment. Animal 2 was in fine condition until the 12th week. At this time the animal began to lose weight and became thin and inactive, but it did not show any lesions of scurvy either when alive or on postmortem examination.

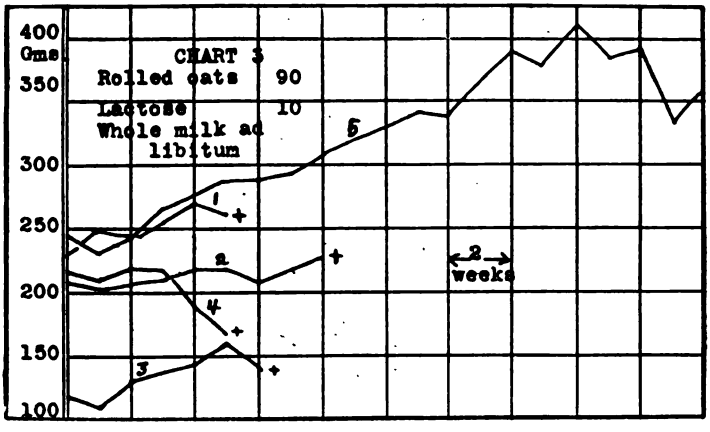


CHART 3. These curves show that 10 per cent of lactose was not as efficient in protecting the animals from scurvy as was 20 per cent. Postmortem examination of Animals 1, 2, 3, and 4 showed lesions of scurvy. Animal 5 grew fairly well throughout the course of the experiment and never showed any signs o having scurvy. It was killed at the end of the 20th week and appeared to be normal in all respects.

Digitized by Google

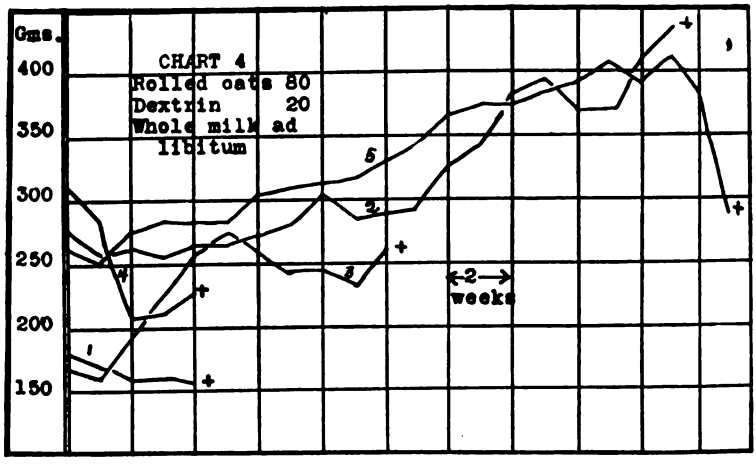


CHART 4. This chart shows the record of animals fed on a ration of rolled oats and dextrin prepared from cornstarch. It is evident by comparing these curves with those of Chart 8 that these animals were benefited to a considerable degree by the ingestion of dextrin in the ration, but they did not do nearly so well as did the animals that received lactose (see Chart 2). All the animals died, but there was a marked difference in the length of time that the animals survived this ration. Animals 2 and 5, which survived the longest, showed the most severe lesions of scurvy. Animal 1 did not show symptoms of scurvy on postmortem examination.

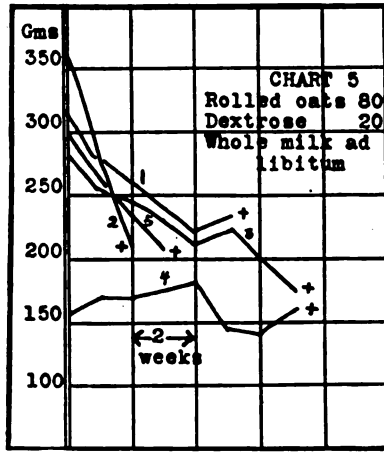


CHART 5. These animals received dextrose in the ration. They did little better than the animals that received no carbohydrate, other than that contained in the oats (see Chart 8). The animals showed severe lesions of scurvy. Two of them, Nos. 4 and 5, suffered with an unusual amount of fermentation. The cecum of all of the animals on this diet was packed with feces that had a very offensive odor.

Digitized by Google

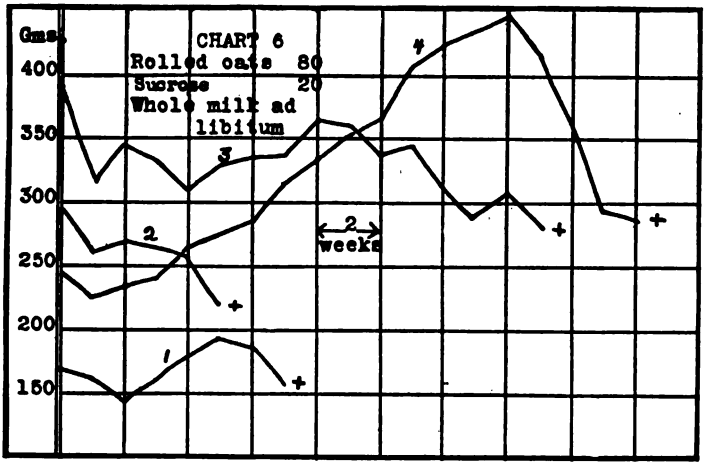


CHART 6. This chart illustrates the behavior of guinea pigs fed on a diet of rolled oats and sucrose and milk. The ingestion of sucrose delayed the onset of the symptoms of scurvy for a considerable time, but eventually the animals all died of scurvy.

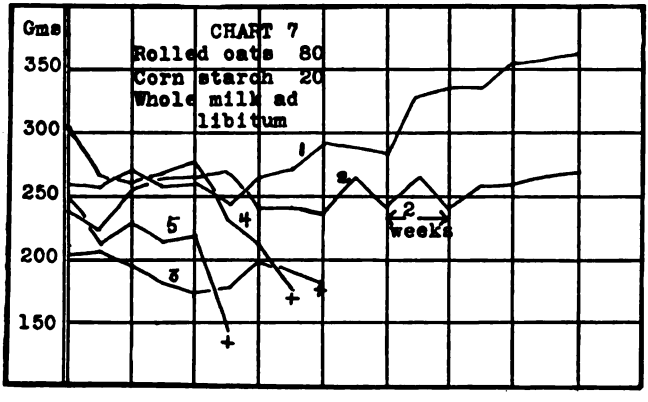


CHART 7. These animals received a ration of rolled oats and cornstarch. The starch afforded considerable protection against scurvy. However, three animals died of scurvy before the 9th week and all showed lesions at this time. Animal 1 is alive at the present writing and is in fair condition. It, however, shows no outward signs of scurvy. Animal 2 shows distinct signs of scurvy, but it is maintaining its weight.

Digitized by Google

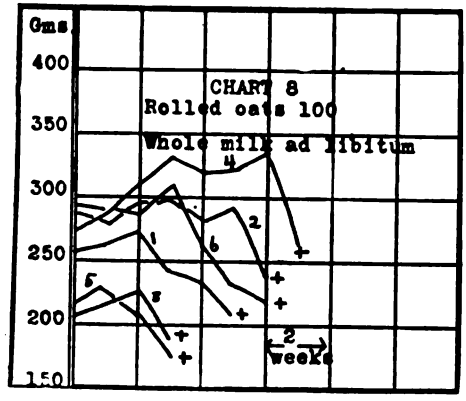


CHART 8. This chart illustrates the typical behavior of guinea pigs fed a ration of rolled oats and whole milk *ad libitum*. The animals generally develop the lesions of scurvy from the 3rd to the 4th week, but a few live longer than this before they show the symptoms. Occasionally an exceptional individual will be found that thrives on this ration. The curve of such an animal was given in our first paper on scurvy (1).

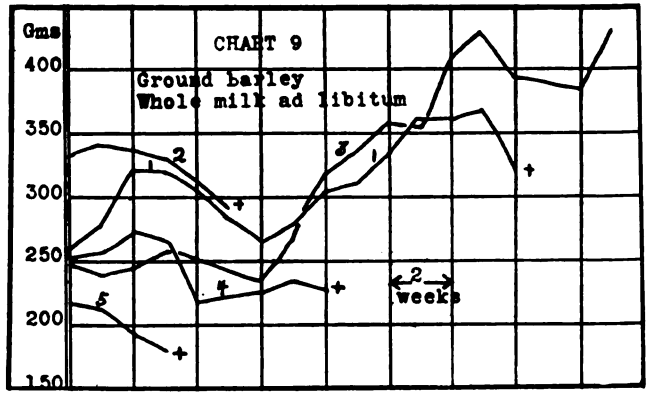


CHART 9. This illustrates the behavior of guinea pigs fed a ration of ground barley and whole milk *ad libitum*. The animals thrive better on this ration than they do on a ration of whole ground oats and milk (see Chart 12). All the animals on this ration, except No. 3, showed signs of scurvy about the 4th week. Animal 3 did not show lesions of scurvy at any time and is in excellent condition at this writing. It should be borne in mind that occasionally a guinea pig will thrive on an oat and milk diet.

Digitized by Google

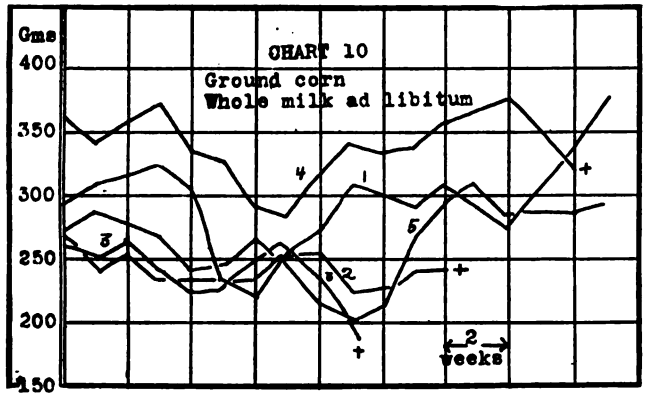


CHART 10. Growth curves of animals fed a ration of corn and milk. The animals did not lose weight as rapidly and they lived longer than did animals receiving barley and milk (see Chart 9). The symptoms of scurvy were not evident in these animals until the 5th and 6th weeks. Animal 5 did not show lesions until the 8th week. Animals 1 and 5 are alive at the present writing No. 1 shows symptoms of mild scurvy, while No. 5 shows more pronounced symptoms.

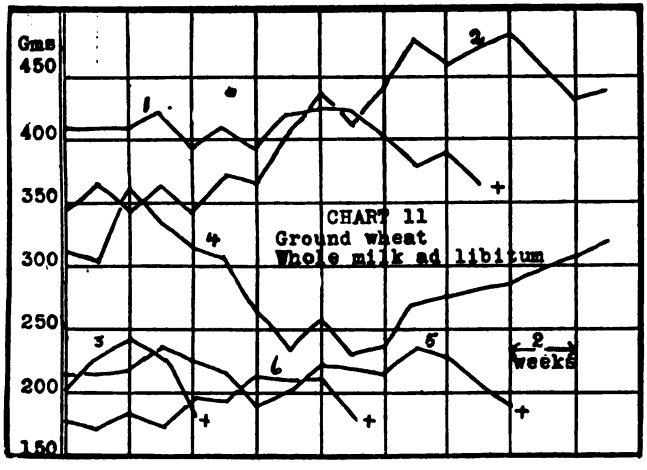


CHART 11. The animals in this group received a ration of whole wheat and milk. These animals grew about as well as did the animals that received corn and milk (see Chart 10). However, the animals in this lot developed lesions of scurvy during the 3rd and 4th weeks, while those on corn developed them the 5th to 6th weeks. Animal 2 never showed any evidence of having scurvy and it remained in excellent condition until the 12th week. Since then it has been losing weight, but it does not show any signs of having scurvy at the present time. Animal 4 developed scurvy the 4th week, and from the 4th to the 8th week it was in a miserable condition. After the 8th week it slowly improved, but its knee joints are still swollen and it walks with difficulty and is very sensitive.

Digitized by Google

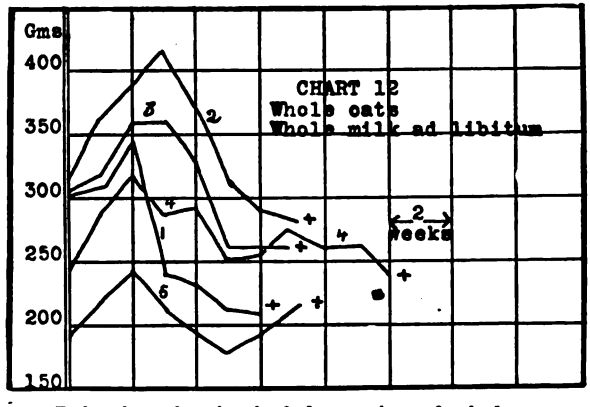


CHART 12. Behavior of animals fed a ration of whole oats and milk All these animals developed scurvy during the 3rd and 4th week. They failed rapidly and all but two died at the end of the 7th week. One animal lived until the 10th week.

Digitized by Google

A CHEMICAL STUDY OF FOOD FISHES.

THE ANALYSIS OF TWENTY COMMON FOOD FISHES WITH ESPECIAL REFERENCE TO A SEASONAL VARIATION IN COMPOSITION.

By ERNEST D. CLARK and LLOYD H. ALMY.

(From the Food Research Laboratory, Bureau of Chemistry, United States Department of Agriculture, Philadelphia.)

(Received for publication, December 11, 1917.)

That a variation in the composition of fish might occur was recognized by Atwater (1) who analyzed fifty-two species, sometimes basing his results on a single specimen, again taking the average of several samples caught at short intervals. Nine species were examined at different seasons of the year. Of these only three species—blackfish, mackerel, and salmon—showed a definite tendency toward a seasonal variation in composition.

A study of the recorded analyses of fish makes it plain that a single examination, even if based on large numbers of individuals, cannot be accepted as representing a typical composition. For example, the herring shows a wide latitude in its gross composition, as may be seen from Table I, where the moist fish ranges from 19.29 to 30.97 per cent of solids, and from 2.41 to 11.01 per cent of fat. This variation in the composition

TABLE I.

The Percentage Composition of Herring, as Determined by Various Investigators.

Fish.	Author.	Water.	Solids.	Fat.	Ash.	Fat-ash-free solids.	Nitrogen.
		per cent	per cent	per cent	per cent	per cent	per cent
Fresh herring.	(See Table IV).	77.10	22.90	2.41	1.66	18.83	3.03
" "	Kostytscheff (3).	76.11	23.89	4.89	1.71	—	—
Little "	Almen (4).	73.22	26.75	5.87	1.65	18.83	3.01
Herring.	Buckland (5).	80.71	19.29	7.11	2.07	—	—
Smoked herring.	König (6).	69.49	30.51	8.51	1.24	21.12	3.38
Fresh "	Payen (7).	70.00	30.00	10.30	1.90	—	—
" "	Atwater (1).	69.03	30.97	11.01	1.50	19.12	3.06

Digitized by Google

of herring has been studied by Hjort (2). It is obvious, therefore, that we should know the locality of the catch, the season of the year, the weight of the fish, and also as much of its habits at the catching time as possible, in order to interpret analytical results.

Polimanti (8) has considered the relation of habitat to the fat content of the flesh and is of the opinion that the deep-sea fish are poor in fat while the surface swimming forms are rich. Aside from the relation between habitat and the content of fat, there is undoubtedly a close relation between the amount of fat stored in the edible portions of the fish and the processes of reproduction. This has been observed by Atwater (1), who gives the composition of ordinary and spent salmon. The fish on their way to the spawning grounds contained 13 per cent of fat, while the spent fish contained but 3 per cent. The work of Miescher-Ruesch (9) on the Rhine salmon gives a similar picture, and the later work of Paton (10) on Scotch salmon, and of Green (11) on Pacific coast salmon, is confirmatory. According to the latter two investigators, the fat stored in the body of the salmon during the period of feeding and growth is the immediate source of energy expended by the fish during spawning migration when no food is taken. Also, the observation by Lichtenfelt (12), who noted that during hunger a fat-rich fish loses a greater proportion of its fat than a fat-poor fish, may, when the analytical data are supplied, throw some light upon the habits of certain species.

Whatever the biological significance of the changing composition may be, the fact remains that a large number of our food fishes have a greatly increased fat content in the late summer and autumn, as shown in Table II, compiled from Atwater's (1) analyses, and also by the analytical results recorded in Table IV.

TABLE II.

Analyses of Food Fish by Atwater, Arranged to Show Seasonal Variation. Fresh Basis.

Fish.		Water.	Solids.	Fat.	Ash.	Nitrogen.
Name.	When caught.					
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
Blackfish.	Mar., 1882	81.36	18.64	0.55	0.65	2.82
"	Apr., 1882	79.64	20.36	0.62	1.03	3.03
"	May, 1882	78.44	21.56	1.44	1.36	3.03
Mackerel.	May, 1880	74.14	25.86	6.99	1.30	2.91
"	Dec., 1880	64.01	35.99	16.30	1.48	3.05

The results of Atwater's investigations have been accepted as representing the correct composition of American food fishes. The dietitian, in calculating the fuel value of the food components present in different species of fish, has used these figures. They have been quoted by a number of writers.

Digitized by Google

In addition to Atwater's classical work on American fishes, the literature contains numerous references bearing on the chemical composition of food fish. The earlier investigations have been carried on mainly by foreign workers, among whom are Schlossberger (13), Morin (14), Weidenbusch (15), von Baumhauer (16), Payen (7), König (6), Buckland (5), Almen (4), Kostytscheff (3), Popoff (17), Sempolowski (18), and Kianizyn (19). The results obtained by most of these authors have been discussed at length by Atwater (1). Their analyses consisted, generally, of determinations of solids, fat, ash, and nitrogen. There has been no concerted analytical study of the composition of American food fish since 1888. European food fishes have been studied more recently, and among the investigations those of Balland (20) and Hollande (21) are especially noteworthy. A comparison of the food value of beef, veal, etc., with fish has been made by Milone (22), Beythien (23), and König and Splittgerber (24).

Methods.

History of Samples.

The fish were all obtained from Philadelphia fish dealers. No fish were purchased of which an accurate history could not be obtained. This history indicated good handling according to present commercial standards and a minimum lapse of time after catching. The analytical work was begun immediately upon the receipt of the sample.

Preparation of Sample for Analysis.

The fish were cleaned by separating as completely as possible from the edible portion the head, tail, skin, entrails, and bones. The edible portion was weighed and the difference between this weight and that of the whole fish represented the refuse, or inedible portion. The flesh was ground in a meat chopper, and the individual samples were mixed thoroughly by hand. As the subsequent analysis requires 2 or 3 days of one analyst's time for completion, it was not convenient to undertake more than one complete analysis of a single species of fish at a time. In the case of large fish but one specimen was used, but of the smaller fish from 2 to 8 were analyzed, the edible portions being united and well mixed before sampling.

Digitized by Google

Methods of Analysis.

Total Solids, Total Nitrogen, Fat, and Ash.—The methods used for the estimation of the quantity of total solids, fat, and ash, were those given in Bulletin No. 107 (revised) of the Bureau of Chemistry, United States Department of Agriculture. Total nitrogen was determined by the method of Kjeldahl and Gunning, using 0.1 N solutions of acid and alkali.

Cold Water-Soluble Nitrogen.—The amount of nitrogenous compounds soluble in cold water was found by grinding 50 gm. of the sample, placing in a 12 ounce wide-necked bottle, adding 240 cc. of water, corking tightly, and shaking on a mechanical shaker. The bottles were removed from the shaker at the end of 15 minutes and the suspended matter was separated by centrifuging for 10 minutes. The opalescent supernatant fluid was poured through a plug of cotton in a funnel. Seven more extractions, each with 240 cc. of water, brought the final volume of the combined filtrate to about 1,900 cc. This solution was transferred to a 2 liter flask and diluted to the mark with water. A second filtration through paper was made when required, though this was usually unnecessary, as the extract could be obtained in most cases free from suspended matter. Aliquots of 50 cc. each were analyzed for nitrogen by the Kjeldahl-Gunning method.

Coagulable Nitrogen.—Portions of 500 cc. of the neutral extract were evaporated to a little less than 250 cc. in 400 cc. beakers. The beakers were placed on wide asbestos gauzes to prevent scorching, and the height of the flame was so regulated that about 2 hours were required to reduce the volume of the solution one-half. The mixture was stirred occasionally to prevent clumps from sticking to the bottom of the beaker. After removing the flame, and while the solutions were still hot, they were always treated with exactly 2 cc. of 20 per cent acetic acid solution to complete the precipitation of the coagulable protein. On cooling, the mixture was transferred to a 250 cc. volumetric flask and diluted to the mark with water. The mixture was filtered and aliquots of 25 cc. each were analyzed for nitrogen.

Hot Water-Soluble Nitrogen.—The flesh residue remaining from the cold water extraction was washed into a 400 cc. beaker with about 250 cc. of hot water. The mixture was boiled for 15 minutes, filtered hot through cotton, the residue again treated with hot water, and boiled for another 15 minutes. The filtrate from the second extraction was added to that of the first and the combined filtrates were transferred to a 500 cc. volumetric flask, cooled, and diluted to the mark with water. This extract was filtered through fine filter paper and nitrogen was determined on 50 cc. aliquots of the filtrate.

Ammonia Nitrogen.—For this determination a modification of the Steel-Gies[1] method was used. 10 gm. of NaCl were dissolved in 100 cc. of water

[1] Steel, M., and Gies, W. J., Some notes on the efficiency of the Folin method for the quantitative determination of urinary ammonia, *J. Biol.*

Digitized by Google

in a tall Folin aeration cylinder. 25 gm. of ground flesh were added and the mixture was shaken until the material was entirely disintegrated. 50 cc. of alcohol were next added to prevent foaming, and 5 cc. of kerosene later if necessary. The mixture was finally treated just previous to aeration with 10 cc. of 10 per cent sodium hydroxide solution. The ammonia was absorbed in dilute sulfuric acid solution (10 cc. of 0.1 N sulfuric acid in 100 cc. of water) in 12 ounce bottles. At the end of a 4½ hour aeration period, the solutions in the absorbing bottle were titrated back with 0.1 N sodium hydroxide solution, using as indicator two drops of 1 per cent alcoholic solution of alizarin red.

Gross Fat Extraction.

In order to obtain the fish oil in a condition as nearly unchanged as possible, care was constantly exercised to prevent overheating of the material. The ground flesh was placed in 6 inch shallow Petri dishes in sufficient quantity to form thin layers when pressed down on the bottoms of the dishes with a spatula. The water was driven off at 50°C. in a vacuum oven. The dried residue was ground, placed in a large paper extraction thimble, and extracted with low boiling petroleum ether (40–50°C.) in a Soxhlet extractor. After a 12 hour extraction, the solvent was evaporated somewhat, the mixture filtered through fine filter paper, the remainder of the ether carefully evaporated, and the oil finally dried for 3 hours at 50°C. in a vacuum oven.

Determination of Fat Constants.

In some fish the amount of fat obtained was so small that but very few of the fat constants could be determined, due to lack of material, and when such a condition existed usually only the iodine number was determined.

Specific Gravity.—The specific gravity was determined in a Sprengel or Ostwald pyknometer at 25°C., compared with water at the same temperature, and the constants were based on weights in air.

Index of Refraction.—This was determined at 30°C. with an Abbe refractometer.

Chem., 1908, v, 71. Steel, M., An improvement of the Folin method for the determination of urinary ammonia nitrogen, *ibid.*, 1910, viii, 365. Shulansky, J., and Gies, W. J., Studies of aeration methods for the determination of ammonium nitrogen. The ammonium nitrogen in beef, *Biochem. Bull.*, 1913, iii, 45. Clark, E. D., Report on meat and fish, 1915, *J. Assn. Agric. Chem.*, 1917, ii, 229.

Digitized by Google

TABLE III.

History of Samples of Fish Analysed.

No. of sample.	Fish.	No. of fish analysed.	Total weight.	When caught	Where caught.	How caught.	How shipped.	Edible portion.
			gm.	*1915*				*per cent*
19	Blackfish (*Tautoga onitis*).	1	3,432	June 16	Lavalette, N. J.	Traps.	Freight, in barrels, iced.	35.1
18	Bonita (*Sarda sarda*).	1	1,833	" 12	Sea Isle City, N. J.	"	" iced.	58.4
33	Cod (*Gadus callarias*).	1	2,475	Nov. 24	Holly Beach, N. J.	Hook.	" in boxes, iced.	49.9
16	Herring (*Clupea harengus*).	8	2,285	June 12	Asbury Park, N. J.	Traps.	" " barrels, iced.	50.5
8	Kingfish (*Menticirrhus americanus*).	3	1,782	May 4	Ocean City, Md.	"	" " " "	46.2
32	Ling (*Urophycis*, sp. (?)).	4	2,339	Nov. 18	Seaside Park, N. J.	Seine.	" " boxes, iced.	42.2
11	Porgy (*Stenotomus chrysops*).	7	2,029	May 14	Block Island, R. I.	"	" " barrels, iced.	37.1
34	Tilefish (*Lopholatilus chamæleonticeps*).	1	5,800	Dec. 17	150 miles off New Jersey shore.	Hook.	" " " "	50.8
14	Silver hake (*Merluccius bilinearis*).	6	2,720	May 26	Lake Como, N. J.	Traps.	" " " "	41.7
9	Bluefish (*Pomatomus saltatrix*).	1	2,769	May 7	Cape Charles, Va.	Hook.	Freight, in barrels, iced.	48.7
27	" " "	1	2,277	Sept. 28	Atlantic Highlands, N. J.	"	" " boxes, iced.	53.7
12	Butterfish (*Poronotus triacanthus*).	7	1,243	May 19	Sea Isle City, N. J.	Traps.	" " " "	44.2
28	" " "	8	1,938	Oct. 12	" " " " "	"	" " " "	52.7
15	Carp sucker (*Carpiodes thompsonii*).	1	1,683	May 29	Sandusky, Ohio (Lake Erie).	"	Express, " " "	33.9
30	" " (" *cyprinus*).	1	4,599	Oct. 20	Delaware Bay.	"	Fish were alive, no ice.	44.1
3	Croaker (*Micropogon undulatus*).	6	1,677	Apr. 10	Buckroe Beach, Va.	Seine.	Freight, in barrels, iced.	30.9
23	" " "	8	1,703	Sept. 8	Chincoteague Island, Va.	Trawl.	" " " "	37.8
6	Flounder (*Pseudopleuronectes americanus*).	2	2,076	Apr. 19	Vineyard Sound.	"	" " " "	34.9
25	" " "	1	2,275	Sept. 22	Long Branch, N. J.	Seine.	" " boxes, iced.	48.4

Digitized by Google

2	Haddock (*Melanogrammus æglefinus*).	2	3,625	Apr. 2	Georges Bank, Mass.	Hook.	Freight, in boxes, iced.	42.9
22	" " "	1	1,365	Aug. 31	" " "	"	" " " "	52.1
5	Striped bass (*Roccus lineatus*).	3	2,775	Apr. 16	Wanchese, N. C.	Gill net.	" " " "	38.7
29	" " " "	7	2,469	Oct. 16	Potomac River.	Hook.	Express, " " "	43.6
10	Sea bass (*Centropristes striatus*).	2	2,562	May 12	Cape May, N. J.	"	" " " "	34.5
24	" " " "	5	1,657	Sept. 14	Anglesea, N. J.	"	" " " "	37.7
17	Spanish mackerel (*Scomberomorus maculatus*).	3	2,658	June 4	Off Boston, Mass.	Seine.	Freight, " barrels, iced.	51.7
31	" " " "	2	2,667	Oct. 26	Race Point, Mass.	"	" " boxes, "	62.7
7	Weakfish (*Cynoscion regalis*).	3	1,339	May 1	Ocean City, Md.	Traps.	Express, " barrels, "	43.8
26	" " "	3	1,922	Sept. 25	Long Branch, N. J.	Seine.	Freight, " " "	54.1
1	Shad (*Alosa sapidissima*) (male).	2	3,988	Apr. 2	Albemarle Sound, Wanchese, N. C.	Seine.	Freight, in boxes, iced.	51.6
4	" " " (female).	1	2,627	" 13	Wanchese, N. C.	"	" " " " "	47.8
13	" " " "	1	1,998	May 22	Port Penn, Del.	Traps.	" " " "	40.1
20	" " " " spent.	2	1,309	June 19	Ship Bottom, N. J.	"	" " barrels, iced.	52.8

Digitized by Google

TABLE IV.

Percentage Composition of Edible Part of Fish.
Fresh Basis.

No. of sample.	Common name of fish.	When caught.	Solids.	Fat.	Ash.	Nitrogen.				
						Total.*	Cold water-soluble.	Coagulable.	Hot water-soluble.	Ammonia.
		1915	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
19	Blackfish.....	June 16	20.0	0.15	1.40	2.93	0.874	0.532	0.241	0.0172
18	Bonita.....	" 12	26.66	1.46	1.71	3.82	1.030	0.389	0.088	0.0251
33	Cod.....	Nov. 24	18.65	0.09	1.23	2.95	0.950	0.530	0.330	0.0170
16	Herring.....	June 12	22.90	2.41	1.66	3.03	1.028	0.516	0.234	0.0177
8	Kingfish.....	May 4	24.60	5.24	1.39	2.83	0.838	0.425	0.172	0.0162
32	Ling.....	Nov. 18	18.30	0.12	1.15	2.69	1.030	0.650	0.240	0.0180
11	Porgy.....	May 14	23.39	2.59	1.37	3.02	1.001	0.620	0.221	0.0165
34	Tilefish.....	Dec. 17	19.66	0.51	1.35	2.80	0.770	0.380	0.220	0.0130
14	Silver hake	May 26	18.86	1.41	1.22	2.60	1.025	0.638	0.211	0.0140
9	Bluefish.....	May 7	23.83	1.54	1.16	3.36	0.760	0.320	0.071	0.0185
27	"	Sept. 28	29.04	8.10	1.11	3.26	0.730	0.360	0.085	0.0180
12	Butterfish.....	May 19	25.66	5.96	1.49	2.89	0.862	0.498	0.020	0.0162
28	"	Oct. 12	30.01	13.52	1.40	2.92	0.850	0.430	0.021	0.0180
15	Carp sucker....	May 29	22.80	2.10	1.20	2.98	1.151	0.787	0.206	0.0154
30	" "	Oct. 20	24.79	4.17	1.20	3.19	1.078	0.748	0.210	0.0170
3	Croaker.....	Apr. 10	20.77	1.25	1.18	2.85	0.813	0.447	0.141	0.0140
23	"	Sept. 8	24.26	3.23	1.37	—	0.880	0.410	0.130	0.0230
6	Flounder.....	Apr. 19	17.54	0.20	1.17	2.56	0.888	0.477	0.117	0.0143
25	"	Sept. 22	21.59	0.37	1.34	2.54	0.740	0.330	0.200	0.0190
2	Haddock.....	Apr. 2	18.32	0.15	1.11	2.33	0.748	0.343	0.147	0.0220
22	"	Aug. 31	20.93	0.09	1.01	2.59	0.800	0.520	0.150	0.0220
5	Striped bass....	Apr. 16	25.70	3.58	1.26	3.21	1.153	0.719	0.102	0.0170
29	" "	Oct. 16	19.83	2.98	1.26	3.07	1.050	0.650	—	0.0166
10	Sea bass.....	May 12	22.02	1.61	1.23	2.98	0.967	0.532	0.106	0.0159
24	" "	Sept. 14	19.44	1.60	1.09	—	0.970	0.700	—	0.0220
17	Spanish mackerel.....	June 4	33.01	12.59	1.20	3.13	0.846	0.458	0.055	0.0185
31	" "	Oct. 26	35.70	16.24	1.11	3.09	0.890	0.570	0.102	0.0240
7	Weakfish.....	May 1	21.41	2.34	1.25	2.83	1.118	0.851	0.203	0.0134
26	"	Sept. 25	19.35	0.52	1.20	—	0.820	0.500	0.290	0.0150
1	Shad (male).....	Apr. 2	35.32	14.43	1.34	3.18	1.112	0.621	0.074	0.0160
4	" (female)...	" 13	34.17	13.93	1.40	3.00	1.147	0.685	0.063	0.0182
13	" " ...	May 22	26.00	5.87	1.29	2.91	0.980	0.549	0.058	0.0191
20	" " spent	June 19	23.38	2.95	1.53	2.98	0.975	0.549	0.182	0.0178

* It is customary to calculate protein as total nitrogen × 6.25. Our knowledge of the proper factor to use with fish proteins is unsatisfactory and pending investigation of this matter it seems best to omit this calculation.

Digitized by Google

TABLE V.

Percentage Composition of Edible Part of Fish.
Calculated on Dry Basis.

No. of sample.	Common name of fish.	When caught.	Fat.	Ash.	Nitrogen.				
					Total.	Cold water-soluble.	Coagulable.	Hot water-soluble.	Ammonia.
		1915	per cent	per cent	per cent	per cent	per cent	per cent	per cent
19	Blackfish	June 16	0.70	6.70	14.02	4.18	2.55	1.15	0.082
18	Bonita	" 12	5.48	6.41	14.33	3.86	1.46	0.33	0.094
33	Cod	Nov. 24	0.46	6.59	15.81	5.09	2.84	1.76	0.091
16	Herring	June 12	10.52	7.25	13.23	4.49	2.25	1.02	0.078
8	Kingfish	May 4	21.32	5.65	11.50	3.41	1.73	0.70	0.066
32	Ling	Nov. 18	0.62	6.27	14.68	5.62	3.55	1.31	0.098
11	Porgy	May 14	11.10	5.85	12.92	4.28	2.67	0.95	0.071
34	Tilefish	Dec. 17	2.59	6.85	14.22	3.91	1.93	1.11	0.066
14	Silver hake	May 26	7.48	6.48	13.80	5.43	3.39	1.42	0.074
9	Bluefish	May 7	6.45	4.87	14.10	3.19	1.35	0.30	0.078
27	"	Sept. 28	27.83	3.81	11.21	2.51	1.24	0.29	0.061
12	Butterfish	May 19	23.27	5.81	11.28	3.36	1.94	0.08	0.063
28	"	Oct. 12	45.02	4.66	9.72	2.83	1.43	0.70	0.059
15	Carp sucker	May 29	9.20	5.27	13.08	5.05	3.45	0.90	0.068
30	" "	Oct. 20	18.05	5.19	13.81	4.35	3.02	0.90	0.074
3	Croaker	Apr. 10	6.02	5.68	13.72	3.91	2.15	0.68	0.067
23	"	Sept. 8	13.30	5.64	—	3.62	1.69	0.54	0.094
6	Flounder	Apr. 19	1.14	6.67	14.60	5.06	2.72	0.67	0.082
25	"	Sept. 22	1.71	6.20	11.76	3.42	1.90	0.90	0.087
2	Haddock	Apr. 2	0.81	6.06	12.72	4.08	1.87	0.80	0.120
22	"	Aug. 31	0.42	4.84	12.43	3.84	2.50	0.72	0.105
5	Striped bass	Apr. 16	13.94	4.90	12.50	4.48	2.80	0.40	0.066
29	" "	Oct. 16	15.01	6.35	15.47	5.39	3.29	—	0.084
10	Sea bass	May 12	7.32	5.59	13.55	4.39	2.42	0.48	0.072
24	" "	Sept. 14	8.22	5.60	—	5.08	3.55	—	0.113
17	Spanish mackerel	June 4	38.14	3.63	9.48	2.56	1.39	0.17	0.056
31	" "	Oct. 26	45.47	3.10	7.38	2.49	1.60	0.29	0.067
7	Weakfish	May 1	10.94	5.85	13.23	5.53	3.98	0.95	0.063
26	"	Sept. 25	2.68	6.19	—	4.63	2.58	1.49	0.077
1	Shad (male)	Apr. 2	40.85	3.80	9.01	3.15	1.768	0.21	0.045
4	" (female)	" 13	40.75	4.10	8.78	3.35	2.005	0.18	0.053
13	" "	May 22	22.58	4.97	11.20	3.77	2.113	0.23	0.074
20	" " spent	June 19	12.62	6.54	12.75	4.17	2.348	0.78	0.076

Digitized by Google

TABLE VI.

Fat Constants of Oils from Fish Flesh.

No. of sample.	Common name of fish.	When caught.	Specific gravity, $\frac{25°}{25}$ C.	Index of refraction 30° C.	Iodine No.	Saponification No.	Acid value.
		1915					
19	Blackfish	June 16	—	—	127.5	—	—
18	Bonita	" 12	0.9389	1.4755	126.8	178.7	28.6
33	Cod	Nov. 24	—	—	94.1	—	—
16	Herring	June 12	0.9292	1.4733	135.4	180.9	13.5
8	Kingfish	May 4	0.9146	1.4663	85.4	195.7	3.4
32	Ling	Nov. 18	—	—	80.8	—	—
11	Porgy	May 14	0.9270	1.4736	136.2	188.4	7.2
34	Tilefish	Dec. 17	—	1.4753	118.8	—	—
14	Silver hake	May 26	0.9397	1.4797	159.0	186.5	10.1
9	Bluefish	May 7	0.9353	1.4749	118.9	185.1	12.4
27	"	Sept. 28	0.9389	1.4760	106.9	—	4.5
12	Butterfish	May 19	0.9213	1.4732	138.2	190.5	3.0
28	"	Oct. 12	0.9177	1.4706	90.2	202.8	—
15	Carp sucker	May 29	0.9232	1.4704	111.7	187.9	7.8
30	" "	Oct. 20	0.9243	1.4700	74.1	240.8	—
3	Croaker	Apr. 10	0.9312	1.4724	115.8	191.2	—
23	"	Sept. 8	—	1.4703	111.5	—	—
6	Flounder	Apr. 19	—	1.4935	130.4	—	—
25	"	Sept. 22	—	1.4820	118.9	197.6	—
2	Haddock	Apr. 2	—	1.4940	133.4	—	—
22	"	Aug. 31	—	1.4907	115.1	—	—
5	Striped bass	Apr. 16	0.9278	1.4748	134.4	196.1	6.4
29	" "	Oct. 16	—	1.4895	89.0	—	—
10	Sea bass	May 12	0.9224	1.4731	132.1	191.7	2.2
24	" "	Sept. 14	—	1.4860	132.2	—	—
17	Spanish mackerel	June 4	0.9201	1.4719	136.7	182.5	3.2
31	" "	Oct. 26	—	1.4730	115.3	204.9	2.6
7	Weakfish	May 1	0.9213	1.4717	120.8	187.0	12.2
26	"	Sept. 25	—	1.4830	104.37	188.4	16.2
1	Shad (male)	Apr. 2	0.9084	1.4709	114.8	191.6	4.6
4	" (female)	" 13	0.9150	1.4710	114.5	190.4	3.5
13	" "	May 22	0.9124	1.4719	121.9	183.7	6.4
20	" " spent	June 19	0.9276	1.4725	125.3	180.6	20.6

Digitized by Google

Iodine Number.—The iodine absorption number was determined by the Hanus Method[2] on 0.1 to 0.2 gm. samples using 25 cc. of iodine solution.

Saponification Number.—Approximately 1.5 gm. of oil were saponified in the usual manner[3] with 25 cc. of alcoholic potash for this determination.

Acid Value.—1 gm. of oil was dissolved in 50 cc. of a neutral mixture of three parts ethyl ether and seven parts 95 per cent alcohol, and titrated with 0.1 N sodium hydroxide solution, using 0.5 cc. of 0.5 per cent alcoholic phenolphthalein solution as indicator. The addition of alkali was continued until one drop produced a pink color which persisted at least 1 minute. The result was calculated to mg. of potassium hydroxide required to neutralize the free fatty acids of 1 gm. of the oil.[3]

Tabulation of Analyses.

It was not possible to obtain samples of each species at varying seasons, hence of some fish but one analysis appears in the tables. It is believed that, aside from their interest in the light of seasonal variation, such analytical data are of value from the composition viewpoint alone. Tables III, IV, V, and VI record the results obtained. The values given are in all cases the average of two or more concordant determinations.

DISCUSSION.

Biological Significance of the Fat Content.

Many sea fish travel in schools, or migrate, in response to stimuli of which we are still ignorant and to regions often unknown at definite times of the year. They feed on small animal organisms, lower plant forms, or other fish, depending upon the habits of the various species. In the contest with fellow members of the school, certain fish will fare better in feeding than the less strong and active ones. This may account for the fact that fish of the same size from the same school occasionally vary considerably in fat content. The fish in Table VII were taken from the same school and show an exceptional degree of variation in this respect.

[2] Hanus, J., Die Anwendung von Jodmonobromid bei der Analyse von Fetten und Oelen, *Z. Untersuch. Nahrungs.-Genussmittel*, 1901, iv, 913.

[3] Official and provisional methods of analysis, *U. S. Dept. Agric., Bureau of Chemistry, Bull. 107* (rev.), 1909, 136.

Digitized by Google

TABLE VII.

Analyses of Weakfish from the Same School. (Caught November 11, 1914.) Composite Samples of Three Fish Each.

No. of sample.	Solids.	Fat.	Ash.	Total nitrogen.
	per cent	*per cent*	*per cent*	*per cent*
1	22.88	1.35	1.16	3.13
2	23.35	2.47	1.10	3.13
3	25.51	4.88	1.16	3.09
4	28.22	8.03	1.12	3.19

A study of Table IV shows that weakfish may contain more fat in the spring than in the early autumn. We know but little of the habits of the weakfish. However, during September we find them full of feed, although the fat content of the fish is extremely low; for example, 0.52 per cent. It may be deduced from this figure that the fish have recently entered upon a feeding period, which assumption is further confirmed by the fact that later in the year—about the middle of November—as much as 8.03 per cent of fat was found (Table VII).

The literature fails to record the analysis of the flesh of the shad before and after its spawning migration. Accordingly, a study of this fish was made, covering a period of about 2 months, during which time the fish had appeared at the mouth of the rivers and had ascended them to fresh water. The later analyses were made at the close of the shad season. The results are shown in Table IV. The most striking differences are those in the fat, this constituent decreasing from 14.43 per cent *before spawning* in April to 2.95 per cent *after spawning* in June. There is likewise a decrease in the water-soluble and coagulable nitrogen, an increase in the specific gravity, iodine number, and acid value of the fats, and a lowering of their saponification numbers.

The shad, like the salmon, does not feed from the time it enters upon spawning migration until it returns to the feeding grounds. Some of the fat is consumed in the tissues to furnish the energy necessary for ascending the rivers, and the remainder is probably transferred to the roe and milt. It would be profitable to extend this investigation to a similar study of the changes in other fish at spawning time.

Digitized by Google

A fundamental relation, which is indicated not only by these results but likewise by the results obtained by Atwater (1), is that as the fat content increases the water content decreases, the protein remaining practically the same. This is strikingly shown in Table VIII in four species of fish whose fat content varies from 0.15 per cent to 14.43 per cent. The fat-free solids, which consist of nitrogenous and mineral constituents, vary but slightly from the mean value of about 21 per cent, showing that the gain or loss in fat is compensated for by a corresponding gain or loss in water rather than protein.

TABLE VIII.

Relation between the Amounts of Fat, Water, and Nitrogen in the Flesh of Fish.

Kind of fish.	Water.	Solids.	Fat.	Fat-free solids.
	per cent	*per cent*	*per cent*	*per cent*
Blackfish	79.10	20.90	0.15	20.75
Bluefish	70.96	29.04	8.10	20.94
Spanish mackerel	66.99	33.01	12.59	20.42
Shad	64.68	35.32	14.43	20.89

A study of the values obtained for the fat constants of the fish oils shows that they are fairly uniform for the different species analyzed. These constants will average, as a rule, approximately as follows:

Specific gravity at $\frac{25}{25}$°C	0.9220
Index of refraction at 30°C	1.4750
Iodine No	110–130
Saponification No	180–190

They are characteristic of fish oils commonly classed with drying oils.

A study of the figures obtained for the acid value of the oils (Table VI) shows that, in general, when fish are low in percentage of fat, the acid value will be high, and when the fat content is high the acid value will be low. This seems to point to the presence in the tissues of fish, in a more or less constant proportion, regardless of the fat content, of an acid substance sol-

Digitized by Google

uble in the fat solvent, or carried out with the fat. Accordingly, when the fat content of the fish is high the acid value is low, because the proportion of this substance to the total fat is low.

The Significance of the Variation in the Distribution of Nitrogen of the Flesh.

An examination of the nitrogen distribution, as given in Table IV, brings out certain facts. It will be observed that not only was the amount of nitrogenous substance soluble in cold water determined, but also the amount extracted by boiling water. The latter solvent was used because of the likelihood of relatively large, and possibly significant, quantities of gelatin-like substances in fish tissues. The results indicate that the amount is more or less constant for fish of the same species but may vary with difference in species. This trend is much more clearly shown in the amount of nitrogenous substances soluble in cold water, where it is seen that the amount of water-soluble nitrogen and coagulable nitrogen is fairly constant for the same fish taken at different times, while the amounts of these substances may be widely different for different species of fish.

The quantity of nitrogen obtained by aeration is generally accepted as indicating the progress of postmortem changes in the composition of flesh, especially those of decay as opposed to ripening changes. The quantities of ammoniacal nitrogen obtained from fresh fish of different species become, therefore, of importance. It must be remembered that the fish used in this study were all very fresh in the market sense of that term. Undoubtedly a greater time between catching and analyzing elapsed in some cases than in others. Additional observations indicate that had all the fish been examined immediately after removal from the water the quantity of this constituent would have been more uniform in the different samples. However, such variations as frequently prevail between the ammoniacal nitrogen in the flesh of the fat-poor and fat-rich tissue cannot be disregarded, nor can they be referred to decomposition changes. A further study of this point is needed. From unpublished data we know that fish packed in ice gradually lose some of their ammoniacal nitrogen owing to the solvent effect of the water. A

Digitized by Google

decomposed fish which has been standing with ice may show a low content of ammoniacal nitrogen for this reason.

The present report deals with the composition of the edible flesh of the fish exclusively. It is realized that a similar analytical study should be made of the viscera of fishes, especially the livers, before attempting to attach to the present data undue biological significance.

BIBLIOGRAPHY.

1. Atwater, W. O., The chemical composition and nutritive values of food fishes and aquatic invertebrates, *Rep. Commissioner of Fish and Fisheries*, 1888, 679.
2. Hjort, J., Fluctuations in the great fisheries of Northern Europe. Conseil permanent international pour l'exploration de la mer, Rapports et Procis-Verbaux, 1914, xx, 1.
3. Kostytscheff, P., The chemical composition of fish products with some remarks on their nutritive value (Russian), see Atwater (1).
4. Almen, A., Analyse des Fleisches einiger Fische, *Mitgeteilt Kgl. Ges. Wiss. zu Upsala*, 1877.
5. Buckland, F., Bestandtheile des Fleisches von Lachsen und Heringen im Vergleich zu dem von Rindfleisch, *Arch. Pharm.*, 1874, series 3, liii, 178.
6. König, J., Der Gehalt der menschlichen Nahrungsmittel an Nahrungstoffen im Vergleich zu ihren Preisen, *Z. Biol.*, 1876, xii, 497.
7. Payen, A., Précis theorique et pratique des Substances Alimentaires, Paris, 4th edition, 1865, 45, 488.
8. Polimanti, O., Über den Fettgehalt und die biologische Bedeutung desselben für die Fische und ihren Aufenthaltsort, sowie über den Fettgehalt je nach dem Alter der Fische, *Biochem. Z.*, 1915, lxix, 145.
9. Miescher-Reusch, F., Statistische und biologische Beiträge zur Kenntniss vom Leben des Rheinlachses im Süsswasser, Internat. Fischerei-Ausstellung, in Berlin, 1880, 154; see also Miescher-Reusch, Die histochemischen und physiologischen Arbeiten, Leipzig, 1897, 116.
10. Paton, D. N., Report of investigations on the life history of the salmon in fresh water, *18th Ann. Rep. Fishery Board of Scotland*, 1898, 143–155.
11. Green, C. W., The storage of fat in the muscular tissue of the king salmon and its resorption during the fast of the spawning migration, *Bull. U. S. Bureau of Fisheries*, 1913, xxxiii, 69.
12. Lichtenfelt, H., Über die chemische Zusammensetzung einiger Fischarten, warum und wie sie periodisch wechselt, *Arch. ges. Physiol.*, 1904, ciii, 353.
13. Schlossberger, Vergleichende chemische Untersuchungen über das Fleisch verschiedener Thiere, Dissertation, Stuttgart, 1840.

Digitized by Google

14. Morin, B., Examen chimique de l'éperlan, *Salmo eperlanus* Linnæus, *J. Pharm.*, 1822, viii, 61.
15. Weidenbusch, H., Über die Analyse des Fisch- und Hühnerfleisch-albumins, und eine neue Bestimmungsmethode des Schwefels, *Ann. Chem.*, 1847, lxi, 370.
16. von Baumhauer, Über die Zusammensetzung des Muskelgewebes der Fische, *J. prakt. Chem.*, 1848, xliv, 506.
17. Popoff, M., Determination of the food value of the most common kinds of fish, (Russian) Dissertation, St. Petersburg, 1882, see Atwater (1).
18. Sempolowski, L., Untersuchungen von Seetieren auf ihren Gehalt an agrikulturchemisch wichtigen Stoffen, *Ber. Landw. Versuchssta.*, 1889, xxxvi, 61.
19. Kianizyn, N. J., Food value of the cod, (Russian) Dissertation, St. Petersburg, 1887, see Atwater (1).
20. Balland, Sur la composition des poissons des crustacés et des mollusques, *Compt. rend. Acad.*, 1898, cxxvi, 1728.
21. Hollande, A. C., Valeur nutritive de la chair des quelques poissons exotiques importés en France durant ces dernières années, *Bull. sc. pharmacol.*, 1913, xx, 405.
22. Milone, U., Composizione, valore nutritivo, ed assimilabilitá della carne muscolare dei pesci, *Boll. Soc. Naturalisti di Napoli*, 1897, x, 311.
23. Beythien, A., Über die chemische Zusammensetzung und den Nährwert verchiedener Fleischsorten, *Z. Untersuch. Nahrungs.-Genussmittel*, 1901, iv, 1.
24. König, J., and Splittgerber, A., Die Bedeutung der Fischerei für die Fleischversorgung im Deutschen Reich, *Landw. Jahrb.*, 1909, xxxviii, Supplement iv, 1.

Digitized by Google

A METHOD FOR THE PREPARATION OF TAURIN IN LARGE QUANTITIES.*

By CARL L. A. SCHMIDT and THOMAS WATSON.

(From the Hearst Laboratory of Pathology and Bacteriology and the Department of Biochemistry of the University of California, Berkeley.)

(Received for publication, January 22, 1918.)

For the preparation of large quantities of taurin for experimental work being carried on in this laboratory, we have found that the abalone, *Haliotis*, which can be obtained in quantity on the Pacific coast, is an excellent source for this substance, giving larger yields than can be obtained from ox bile, the more common source for taurin.[1] Kelly[2] found that tanriu was present in *Pecten opercularis* and *Mytilus edulis*, and it was likewise found by Mendel[3] in the muscle of *Haliotis*.

Miyake, working in this laboratory with Takeoka,[4] prepared taurin from the abalone. His process was to precipitate the proteins in the aqueous extract obtained by pressing out the juice from the finely ground and boiled abalone muscle, by the addition of potassium aluminum sulfate, filtering, and removing sulfates by barium hydroxide. The excess of the latter was removed by carbon dioxide precipitation and the taurin repeatedly crystallized.

We have used Miyake's method a number of times and found that it was not the most economical and suitable method when large amounts of material are to be used. The difficulties of filtering and washing the enormous precipitate of barium sulfate

* Work aided by a grant to the Medical Research Committee from the State Council of Defense, and the George Williams Hooper Foundation for Medical Research.

[1] Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460. Tauber, S., *Beitr. chem. Physiol. u. Path.*, 1903, iv, 323.

[2] Kelly, A., *Beitr. chem. Physiol. u. Path.*, 1904, v, 377.

[3] Mendel, L. B., *Beitr. chem. Physiol. u. Path.*, 1904, v, 582.

[4] Takeoka, M., *J. Infect. Dis.*, 1917, xx, 442.

Digitized by Google

led us to look for an easier method. In our hands the method described below has given better yields of taurin than that used by Miyake and has enabled us to work with larger quantities of material at less expense. Several kilos of taurin have been prepared by this method.

After removal of the shell and intestinal tract of the abalone, the muscle is finely ground, heated in a steam sterilizer for a number of hours, and the juice removed by pressure. The resultant cake is reground and again extracted by addition of distilled water and heating. To each 13 liters of the combined extracts is added sufficient glacial acetic acid (about 15 cc.) to make the concentration 0.02 N. This gives the maximum precipitate of protein. After heating for several hours the liquid is transferred to settling bottles, the clear supernatant liquid syphoned off, and the remainder filtered. To the combined filtrates 100 cc. of strong hydrochloric acid are added, the mixture is again heated, and a small additional precipitate removed as before. The clear liquid is evaporated to about one-third of its volume, 500 cc. of strong hydrochloric acid are added, and the mixture boiled until a test sample shows no precipitation of protein on addition of several volumes of alcohol. The liquid is further concentrated and taurin precipitated by the addition of sufficient alcohol to give a concentration of 60 to 70 per cent. On cooling, large amounts of taurin crystals separate, which are filtered and washed with 60 per cent alcohol. The taurin is further purified by boiling with charcoal, crystallizing from water, and washing the crystals with small amounts of water until free from sulfates. Determination of sulfur gave theoretical values. Eight dozen abalones (74 kilos of muscle) gave a yield of 340 gm. of taurin.

An additional yield of 22 gm. was obtained by precipitating the sulfates in the mother liquor by addition of barium hydroxide and crystallizing the taurin. This gave a total yield of 362 gm.

Digitized by Google

ON THE ELIMINATION OF TAURIN ADMINISTERED TO MAN.*

By CARL L. A. SCHMIDT, EDWARD VON ADELUNG, AND THOMAS WATSON.

(From the Hearst Laboratory of Pathology and Bacteriology and the Department of Biochemistry of the University of California, Berkeley.)

(Received for publication, January 22, 1918.)

Apparently the only experiments concerned with the elimination of taurin administered to man were carried out by Salkowski.[1] He found that taurin taken by mouth was eliminated to the extent of about 87 per cent in the urine, but none of the sulfur appeared in the oxidized form. In fact, the greater portion of the taurin was eliminated as taurocarbamic acid. Symptoms of diarrhea followed the ingestion of 5 gm. doses of taurin. Taurin was found by Salkowski to be toxic for rabbits when given by mouth.

In connection with other experiments being carried out in this laboratory it was desirable to study further the elimination of taurin when administered in various ways to man. For this purpose subjects were placed on simple constant diets and sulfur determinations made in periods preceding and following the administration of taurin. Ringer's solution containing about 6 per cent of taurin was used for the injections. The results obtained are summarized in the tables which follow.

A perusal of the tables shows that the increase of urinary sulfur following the administration of taurin occurs almost entirely in the neutral sulfur fraction, although there is a slight but unmistakable rise in inorganic sulfur following the administration

* Work aided by a grant to the Medical Research Committee from the State Council of Defense, and the George Williams Hooper Foundation for Medical Research.

[1] Salkowski, E., *Virchows Arch. path. Anat.*, 1873, lviii, 460; *Ber. chem. Ges.*; 1872, v, 637.

Digitized by Google

TABLE I.

Subject 1.

Day of experiment.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total sulfur.	Remarks.
	gm.	gm.	gm.	gm.	
1	0.382	0.077	0.071	0.530	
2	0.410	0.084	0.045	0.540	
3	0.424	0.083	0.058	0.566	
Averages.....	0.406	0.081	0.058	0.545	
4	0.483	0.050	1.023	1.557	5 gm. of taurin (S = 1.285 gm.) taken by mouth.
5	0.439	0.062	0.090	0.592	
6	0.430	0.067	0.084	0.581	
7	0.385	0.072	0.079	0.536	

TABLE II.

Subject 2.

Day of experiment.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total sulfur.	Remarks.
	gm.	gm.	gm.	gm.	
1	0.473	0.032	0.092	0.597	
2	0.428	0.040	0.101	0.568	
3	0.398	0.053	0.088	0.538	
Averages.....	0.433	0.042	0.093	0.568	
4	0.398	0.042	0.489	0.928	3 gm. of taurin (S = 0.771 gm.) intravenously.
5	0.428	0.029	0.339	0.796	
6	0.437	0.042	0.065	0.544	
7	0.450	0.070	0.061	0.582	

of taurin by mouth, as shown by Subjects 1 and 3, showing that slight oxidation has taken place. The taurin is almost entirely eliminated within the 24 hours following the administration, although a slight rise above the normal is still apparent in the 2 following days. This is especially noticeable in the case of Subject 2. For Subjects 1 and 2 a recovery of 86 and 75 per cent respectively of the taurin sulfur was obtained in the urine. These figures are based on the assumption that the average values obtained in the foreperiod can be taken as a constant for the 3 days following the taurin administration. Owing to a greater varia-

Digitized by Google

TABLE III.

Subject 3.

Day of experiment.	Inorganic sulfur.	Ethereal sulfur.	Neutral sulfur.	Total sulfur.	Remarks.
	gm.	*gm.*	*gm.*	*gm.*	
1	0.448	0.031	0.057	0.544	
2	0.404	0.029	0.099	0.531	
3	0.419	0.042	0.130	0.591	
Averages.....	0.424	0.034	0.095	0.555	
4	0.439	0.035	1.751	2.225	10 gm. of taurin (S = 2.48 gm.) subcutaneously.
5	0.444	0.044	0.132	0.619	
6	0.499	0.053	0.101	0.652	
7	0.706	0.044	1.238	1.988	10 gm. of taurin (S = 2.48 gm.) by mouth.
8	0.516	0.048	0.127	0.692	
9	0.468	0.029	0.119	0.615	
10	0.417	0.030	2.006	2.453	10 gm. of taurin (S = 2.63 gm.) intravenously.
11	0.492	0.031	0.202	0.725	
12	0.560	0.026	0.152	0.739	
13	0.535	0.032	0.109	0.676	

tion in the sulfur content of the diet of Subject 3 the sulfur output for the 3rd day following the taurin administration is taken as the normal value for that period. This gives a percentage recovery of the taurin sulfur in the urine, when given subcutaneously, by mouth, or intravenously, as 62, 59, and 72 per cent respectively. These figures compare favorably with those reported by Salkowski. Pending the development of an easy method for determining the forms of neutral sulfur on which work is under way, we give no results as to the amounts and nature of the compounds by which taurin is excreted in the urine.

It should also be noted that contrary to the findings of Salkowski, no symptoms which could be attributed to the administration of taurin were shown by the human subjects with the doses given, or even when larger amounts were employed. Large amounts may also be given intravenously to rabbits without toxic effect.

Digitized by Google

Digitized by Google

SYNTHESIS AND OXIDATION OF TERTIARY HYDROCARBONS.

By P. A. LEVENE and L. H. CRETCHER, Jr.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 31, 1918.)

The present investigation was evolved from the previous work on the structure of branched chain fatty acids. Levene and Allen[1] have called attention to the lack of a convenient method for determining the location of the tertiary carbon atom in the branched chain fatty acids. An attempt was made to solve the problem by the oxidation of the fatty acids themselves in the hope that the tertiary carbon atom, as the one most susceptible to oxidation, might prove to be the point of disruption of the carbon chain. It is still possible that the solution of the problem may be reached by this mode of attack. Experience has shown, however, that the oxidation of any fatty acid takes place at more than one point of its carbon chain. Because of this the products of oxidation are numerous and it is not always an easy task to formulate the structure of the original molecule on the basis of many fragments. Hence it was thought that an advantage might be gained if prior to oxidation the molecule of the acid could be so transformed as to possess fewer points susceptible to the action of oxidizing agents. It was natural to think in this connection of the hydrocarbons, since every fatty acid is readily convertible into the corresponding hydrocarbon.

Can tertiary hydrocarbons be oxidized by a permanganate solution, or by some other oxidizing agent? The literature on the subject is very meager. In 1901, Zelinsky and Zelikow[2] made the observation that 3-methylpentane is readily oxidized by means of potassium permanganate. Prior to that observa-

[1] Levene, P. A., and Allen, C. H., *J. Biol. Chem.*, 1916, xxvii, 433.

[2] Zelinsky, N., and Zelikow, J., *Ber. chem. Ges.*, 1901, xxxiv. 2865.

Digitized by Google

tion it was universally accepted that saturated aliphatic hydrocarbons are not attacked by this reagent.

The observation of the Russian chemists has not been followed up by a study of the products of oxidation, nor has it been extended to observations on other hydrocarbons. The present work was undertaken with the object of obtaining the lacking information.

Since few tertiary hydrocarbons are readily accessible the work naturally fell into two parts, one directed towards the synthesis of tertiary aliphatic hydrocarbons and the other to the study of their behavior towards potassium permanganate. The work is reported in its present incompleteness because one of the authors has accepted a commission with the United States Army.

The most practical and economical way for the preparation of the hydrocarbons was found to be the one based on the reduction of acids obtained by the malonic ester synthesis. In a general way the routine adopted in the work of Levene and Allen was also followed here. However, a marked improvement was introduced in the method of the reduction of esters to the corresponding alcohols. The details are given in the experimental part. Up to the present there were prepared all the intermediate substances leading up to 2-butylhexane as well as the hydrocarbon, also all the intermediate products leading up to 4-butyloctane but not the hydrocarbon.

Regarding the behavior of 2-butylhexane towards permanganate it was found that it readily underwent oxidation in an alkaline solution of the reagent. However for the purpose of the study of the products of oxidation, special conditions had to be chosen. Namely, it was found that when the oxidation was permitted to proceed at moderately elevated temperatures (between 80–90°C.), the only oxidation products that could be detected were formic and carbonic acids. On the other hand when the oxidation was conducted at a temperature in the neighborhood of 25°C. evidence was obtained of the formation of butyric acid. This acid was identified as its silver salt. The present experiment was carried out only on a small sample of material. It is intended to continue the work on a larger scale.

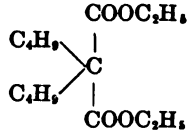
The experience gained up to the present is important in as far as it indicates the conditions of experimentation which will per-

Digitized by Google

mit the isolation of intermediate products of oxidation of the tertiary hydrocarbons.

EXPERIMENTAL.

Diethyl Dibutylmalonate.



This ester was obtained by the action of butyl iodide and sodium ethylate upon diethyl malonate. One molecule of sodium ethylate and one of butyl iodide were added to malonic ester and the mixture was boiled until neutral. After the first substitution was complete another molecule of sodium ethylate and butyl iodide were added and the mixture was again boiled on the water bath until it was no longer alkaline to litmus. The attempt to make both substitutions at the same time, *i.e.* by heating the malonic ester with two molecules of sodium ethylate and of butyl iodide, gave unsatisfactory results. Diethyl dibutylmalonate boils at 153–154° at 14 mm. (corrected).

0.1006 gm. substance gave 0.2464 gm. CO_2 and 0.0938 gm. H_2O.

	Calculated for $C_{15}H_{28}O_4$:	Found:
C	66.58	66.80
H	10.29	10.42

Dibutylmalonic Acid.

This substance was prepared by the saponification of the corresponding ester in the following manner. Malonic ester was converted into diethyl dibutylmalonate as described above. For the preparation of this acid in quantity the ester was not isolated, but to the alcoholic solution of the ester resulting from the treatment of malonic ester with sodium and butyl iodide there was added a solution of potassium hydroxide in the minimal amount of water. The solution was boiled on the water bath under a reflux for 10 hours to complete the saponification, the mixture was then transferred to a large beaker, the alcohol

Digitized by Google

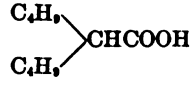
evaporated, a small amount of water was added, and the acid liberated by the addition of concentrated hydrochloric acid to the cooled solution. The acid separated in solid form. It may be recrystallized from benzene from which solvent it separates in long, transparent, prismatic needles. It is slightly soluble in water but practically insoluble in concentrated salt solution.

114 gm. of this dibasic acid were obtained from 100 gm. of malonic ester, corresponding to a yield of 84 per cent of the theory. Dibutylmalonic acid melts at 163° with slight decomposition.

0.1461 gm. substance gave 0.3260 gm. CO_2 and 0.1199 gm. H_2O.

	Calculated for $C_{11}H_{20}O_4$:	Found:
C	61.15	60.85
H	9.20	9.19

2-Butylhexylic Acid.



The following will describe a typical experiment by which this acid was prepared. 120 gm. of dibutylmalonic acid were heated in a distilling flask to 180°. This temperature was maintained until the carbon dioxide was no longer evolved. The liquid remaining in the flask was then distilled. At atmospheric pressure 2-butylhexylic acid boils at 255° (corrected); at 16 mm. the boiling point is 153°. The specific gravity at 16° is 0.899. The yield was 90 gm.

0.1681 gm. substance gave 0.4285 gm. CO_2 and 0.1711 gm. H_2O.

	Calculated for $C_{10}H_{20}O_2$:	Found:
C	69.76	69.51
H	11.62	11.39

Ethyl 2-Butylhexylate.

130 gm. of the corresponding acid were boiled for 8 hours with four molecules of absolute alcohol and a small amount of sulfuric acid. The yield of ester was 144 gm. Ethyl 2-butylhexylate boils at 114–115° at 15 mm. (corrected).

Digitized by Google

0.1004 gm. substance gave 0.2652 gm. CO_2 and 0.1060 gm. H_2O.

	Calculated for $C_{12}H_{24}O_2$.	Found:
C	72.00	71.98
H	12.00	11.85

2-Butylhexyl Alcohol.

The method used for the reduction of ethyl 2-butylhexylate to 2-butylhexyl alcohol was essentially the same as that described by Levene and Allen. However, it has been found, in this instance, that the time of reduction can be reduced to half providing the following two changes in procedure are observed: The mixture of alcohol and ester should be added so slowly that no initial cooling is required. That stage of the reaction having been reached when the addition of alcohol ceases to cause more refluxing, the remainder of the alcohol necessary to affect complete solution of the sodium is rapidly added. In this way the reduction may be completed in about 25 minutes and the yield in no way impaired. The yield of alcohol corresponds to between 65 and 70 per cent of that theoretically possible. The acid resulting from saponification was recovered after the manner described by Levene and Allen. The alcohol boils at 218–219° (corrected). Its specific gravity is 0.836.

0.1447 gm. substance gave 0.4044 gm. CO_2 and 0.1802 gm. H_2O.

	Calculated for $C_{12}H_{26}O$:	Found:
C	75.95	76.14
H	13.92	13.93

2-Butylhexyl Iodide.

2-Butylhexyl alcohol was boiled for 5 hours with three molecules of constant boiling hydriodic acid. The yield of iodide was about 80 per cent. The boiling point is 124–125° at 13 mm. (corrected). The specific gravity is 1.267.

0.2242 gm. substance gave 0.1956 gm. AgI.

Calculated for $C_{10}H_{21}I$:	Found:
47.38	47.16

Digitized by Google

2-Butylhexane.

2-Butylhexane was prepared by the reduction of the corresponding iodide with zinc and glacial acetic acid. 75 gm. of iodide were mixed with 350 cc. of glacial acetic acid and allowed to stand 3 days on the water bath under a reflux. 60 gm. of powdered zinc were added in small amounts. On addition of water two layers were formed; the upper layer of hydrocarbon, after refractionation, weighed 25 gm. The boiling point of 2-butylhexane is 165° (corrected). The specific gravity is 0.738.

0.1010 gm. substance gave 0.3726 gm. CO_2 and 0.1394 gm. H_2O.

	Calculated for $C_{10}H_{22}$:	Found:
C	84.51	84.41
H	15.49	15.41

Diethyl 2-Butylhexylmalonate.

90 gm. of malonic ester (an excess of 20 per cent above the theory) were converted into the monosodium derivative by the addition of 10.6 gm. of sodium in alcohol; 125 gm. of 2-butylhexyl iodide were slowly added and the mixture was boiled on the water bath until neutral. The yield of the substituted ester was 90 gm. This ester boils at 180° at 14 mm. (corrected).

0.1064 gm. substance gave 0.2664 gm. CO_2 and 0.0972 gm. H_2O.

	Calculated for $C_{17}H_{32}O_4$:	Found:
C	68.00	68.29
H	10.60	10.23

2-Butylhexylmalonic Acid.

This acid was prepared from the corresponding ester by saponification with potassium hydroxide in alcohol solution. It crystallizes from low boiling petroleum ether in transparent, rhombic needles which melt at 88° (corrected).

0.1018 gm. substance gave 0.2384 gm. CO_2 and 0.0876 gm. H_2O.

	Calculated for $C_{13}H_{24}O_4$:	Found:
C	63.93	63.87
H	9.83	9.63

Digitized by Google

4-Butyloctylic Acid.

The method used for the preparation of this acid was exactly analogous to that described for the preparation of 2-butylhexylic acid. The boiling point is 173–174° at 12 mm. (corrected). The specific gravity is 0.901.

0.1034 gm. substance gave 0.2736 gm. CO_2 and 0.1108 gm. H_2O.

	Calculated for $C_{12}H_{24}O_2$:	Found:
C	72.00	72.16
H	12.00	12.00

Ethyl 4-Butyloctylate.

In the preparation of this substance 36 gm. of 4-butyloctylic acid were boiled for 6 hours with four molecules of absolute alcohol and a few drops of sulfuric acid. The refractionated product weighed 32 gm. It boils at 139° at 10 mm. (corrected).

0.1000 gm. substance gave 0.2692 gm. CO_2 and 0.1104 gm. H_2O.

	Calculated for $C_{14}H_{28}O_2$:	Found:
C	73.67	73.41
H	12.24	12.35

4-Butyloctyl Alcohol.

28 gm. of the above ester upon reduction yielded 14 gm. of alcohol. The boiling point is 139° at 15 mm. Its specific gravity is 0.841.

0.1266 gm. substance gave 0.3584 gm. CO_2 and 0.1266 gm. H_2O.

	Calculated for $C_{12}H_{26}O$:	Found:
C	77.29	77.20
H	13.57	13.90

4-Butyloctyl Iodide.

This iodide was prepared by boiling a mixture of 10 gm. of the corresponding alcohol with four molecules of constant boiling hydriodic acid. The yeild of iodide was 14 gm. It boils at 143° at 8 mm. Specific gravity, 1.194.

Digitized by Google

0.1992 gm. substance gave 0.1566 gm. AgI.

	Calculated for $C_{12}H_{25}I$:	Found:
I	42.90	42.32

Oxidation of 2-Butylhexane.

The hydrocarbon was oxidized by allowing it to stand in contact with an alkaline solution of potassium permanganate at room temperature for about 4 weeks. Three equivalents for permanganate were used, enough water being added to make the concentration of the permanganate 5 per cent at the beginning of the oxidation. Only a small amount of the hydrocarbon was oxidized. The unoxidized portion was separated from the aqueous layer, the latter acidified and distilled with steam into diluted ammonia. This distillate was concentrated to a small volume *in vacuo* and the fatty acid precipitated as the silver salt.

0.0971 gm. silver salt gave on ignition 0.0531 gm. silver.

	Calculated for $C_4H_7O_2Ag$:	Found:
Ag	55.38	54.68

Digitized by Google

COPPER-PHOSPHATE MIXTURES AS SUGAR REAGENTS.

A QUALITATIVE TEST AND A QUANTITATIVE TITRATION METHOD FOR SUGAR IN URINE.

By OTTO FOLIN and W. S. McELLROY.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

(Received for publication, January 25, 1918.)

In connection with a previous investigation[1] it was observed that alkaline phosphates can hold copper hydroxide in solution. So far as we know phosphates have never been used as a solvent for copper hydroxide in the preparation of reagents for sugar, yet it was thought that phosphates might possess distinct advantages for these purposes. They are less expensive than tartrates, citrates, or glycerol, they do not reduce sugar, and they tend to regulate the degree of alkalinity at a lower level of hydroxyl ion concentration than is obtained by carbonates alone; a given amount of sugar should consequently reduce more copper in such solutions.

The serviceability of phosphates in the preparation of copper reagents for sugar was soon demonstrated, but they can be used in so many different ways that it has been rather difficult to decide which combination to adopt. (Potassium salts have been excluded on account of their cost.)

Qualitative Test for Sugar in Urine.—One good copper-phosphate reagent is made as follows: Dissolve 100 gm. of sodium pyrophosphate (U. S. P.), 30 gm. of crystallized disodium phosphate, and 50 gm. of anhydrous sodium carbonate, in about 1 liter of water. To this solution add 13 gm. of copper sulfate previously dissolved in about 200 cc. of water. The solution seems to keep indefinitely, except that in cold weather some phosphate may crystallize out. The reagent should therefore not be left in very cool places, although it makes little or no difference whether a small part of

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 477.

Digitized by Google

the phosphate has formed a crystalline cake in the bottom of the container.

The solution is used exactly as is Benedict's reagent for sugar: To about 5 cc. in a test-tube add 5 to 8 drops of urine (not over 0.5 cc.) and boil 1 minute, or heat in boiling water for 3 to 5 minutes. Minute traces of sugar are indicated by various grades of turbidity, as in Benedict's test; larger amounts, by unmistakable cuprous oxide precipitates. The test is quite as sensitive and reliable as Benedict's and a trifle more prompt. The only point of distinction which should be noted in the use of this reagent is that unless a very marked turbidity is noted in the hot solutions the result must be regarded as clinically negative. The slight turbidity occurring after cooling represents only the reducing action of normal urine.

Quantitative Titration of Sugar in Urine.—The quantitative method described below for the titration of sugar in urine we believe to be practical and inexpensive.

The only solution required is an acidified copper sulfate solution containing 60 gm. of $CuSO_4.5HO_2$ per liter.[2] 5 cc. of this solution correspond to 25 mg. of dextrose or levulose,[3] 45 mg. of anhydrous maltose, or 40.4 mg. of anhydrous lactose. The other necessary reagent is a dry mixture containing 100 gm. of disodium phosphate crystals ($HNa_2PO_4.12H_2O$), 60 gm. of dry sodium carbonate ($Na_2CO_3.H_2O$), and 30 of sodium or potassium sulfocyanate. These salts are mixed together in a large mortar;

[2] Pure copper sulfate solutions gradually decompose slightly giving a sediment of copper hydroxide, or silicate. This decomposition is presumably caused by the solvent action on the glass. To prevent it add about 2 cc. of concentrated sulfuric acid for each 30 gm. of copper sulfate used, in making the standard 6 per cent copper sulfate solution.

[3] In titrations of levulose the oxidation is so rapid and complete that practically no attention need be paid to the time factor, so important in the titration of other sugars. A levulose titration can be finished in about 2 minutes.

The dextrose and levulose used were Kahlbaum preparations giving correct polariscope values. The milk sugar was purified by recrystallization from dilute alcohol and acetone. The maltose was a sample of Kahlbaum's, but contained traces of dextrin. It was purified by dialyzing for 3 hours, and the maltose value determined by the polariscope in the dialysate was accepted as representing pure maltose. We have not been able to obtain any strictly pure maltose.

Digitized by Google

the mixture keeps indefinitely.[4] Less than 1 gm. of the sulfocyanide, now so difficult to obtain, is consumed for each titration.

The titrations are made in test-tubes. By this innovation the following advantages are gained: (1) the cost of chemicals is greatly reduced; (2) the preliminary heating period is very short; (3) there is no need for regulating the flame to some definite speed of boiling as this is accomplished by moving the test-tube sidewise through the flame; (4) the disappearance of the last traces of blue color is more sharply marked because the volume of liquid is small; (5) there is little or no return of any blue or green color at the end of the titration. Independently of the shape of vessel used for the titration the return of a blue or greenish color by reoxidation is very slow in our titration mixtures. Using pure sugar solutions and test-tubes the titrated solutions remain colorless for several hours.

The titrations are made on undiluted urine even when as much as 6 or 7 per cent of sugar is present. This simplification is made possible by attaching to the tip of an ordinary 25 cc. glass-stoppered burette another tip, consisting of a glass tube drawn out at one end to an almost capillary bore. It is a very simple matter, and requires only a few minutes' work, to make a dozen such tips which will deliver between 45 and 55 drops of urine per cc. We have found these fine tips very helpful in many other kinds of accurate titrations; they permit the measurement of a fraction of 1 cc. with almost as great an accuracy as the measurement of 5 cc. with an ordinary burette.

The burettes carrying accessory tips are most conveniently filled by suction instead of by pouring in the urine from the top. This mode of filling the burette avoids all spilling of urine, all foaming within the burette, and all waiting for the meniscus to reach the proper level. The filling of the burette by suction has also the advantage that it eliminates the necessity of rinsing the burette with the sugar solution or urine to be titrated. A 5 per cent urine can be used directly after a 0.5 per cent one, or *vice versa*. That this is the case can be shown by filling the burette with water after it has just been emptied from a urine containing

[4] When first made the salt mixture tends to cake or set a trifle. If it is left in the mortar or other vessel, covered with paper, over night and is then stirred up once more, before bottling, it does not harden again.

Digitized by Google

5 per cent of sugar and then testing the water in the lower part of the burette for sugar. It contains no sugar. For the suction we use a rubber tube about 30 cm. long, having a glass tip at the lower end; we attach it to the burette by means of a small rubber stopper and a bent glass tube; since the burette needs no cleaning or rinsing the suction arrangements need seldom be removed. The rubber tube can of course be omitted. The essential point is merely to fill the burette, like a pipette, from the bottom by means of suction.

Sugar Titration.

Fill the burette as described (by suction) to the zero mark. Into a test-tube introduce a pebble, 5 cc. of the copper sulfate solution (corresponding to 25 mg. of dextrose), and 4 to 5 gm. of the dry salt mixture. Shake and heat until a clear solution is obtained; this takes usually only a few seconds. Add 25 drops of the urine and boil *very gently* for 2 minutes. If all the copper has been reduced the urine contains probably more than 5 per cent sugar. In that case the determination must be started over again. If the copper is but slightly reduced, yielding only a small amount of white precipitate, add more urine, from 10 to 25 drops depending on the amount of unreduced remaining copper, and boil *gently* for another minute. If at the end of the second boiling period most of the copper has been reduced, complete the titration by the drop system, keeping count of the number of drops added and boiling 1 minute after each subsequent addition of urine. At the end of the titration determine how many additional drops of urine are required to give a volume of 1 or 2 cc. For example, if 37 drops were used in reducing the copper another 15 drops may be required to give a volume of 1 cc. and the volume of urine taken is therefore $\frac{37}{52}$ of 1 cc. Or if 70 drops were used and 36 more are needed to give a volume of 2 cc., then the volume of urine used is $\frac{70}{53}$ of 1 cc. If 50 drops of urine have not reduced most of the copper, then the titration is finished by the addition of larger amounts of urine, without counting the drops, and at the end of the titration the volume of urine taken is determined, as in any other titration, by the reading on the burette. 2.5 divided by the volume of urine

Digitized by Google

taken, whether this is several cc. or a fraction of 1 cc., gives the per cent of sugar in the urine. It is, of course, desirable to confirm the results of the titration by a repetition. In this case the first addition of urine should be only 2 or 3 drops less than the full amount of urine required and the first boiling period should be 3 minutes. The titration is then finished by boiling 1 minute after each subsequent addition of urine. The total boiling period must not be less than 4 minutes, and if it is more than 7 the results obtained tend to be a trifle high. 5 to 6 minutes is the time for which the copper value has been adjusted.

For accurate work two precautions must be observed in connection with the drop system of sugar titration. The drops should be delivered at a slow rate of speed, not faster than 1 drop per second. And the portion of the burette most used, the first 2 cc., should be calibrated. (1 cc. of distilled water weighs about 997 mg.) After the first 2 cc. of the burette have been calibrated by weight the remaining parts of the burette can be calibrated in a few minutes by means of the drop system.

Special Burette for Sugar Titrations.—While very satisfactory results are obtained in the titration of undiluted sugar urines by means of ordinary burettes and the drop system described in the preceding section, we recognize that many who do or teach sugar titrations will prefer to pay for fine burettes and thus do away with the necessity of keeping count of the number of drops used in each titration. Further, a small burette has the advantage that two or more titrations can be made with as little as 10 cc. of urine or sugar solution—an important consideration when teaching large classes, and also for certain kinds of work, as, for example, for the titration of sugar in human milk. We recommend for this work 5 cc. burettes with glass stop-cocks graduated in 0.02 of a cc.[5] These burettes can be read to within 0.01 of 1 cc.

These burettes must be filled by suction. The tips of the burettes are necessarily too coarse to deliver more than 25 or 30 drops per cc. The fine accessory tips delivering 45 to 55 drops per cc. already described are therefore highly desirable in connection with these burettes. In other words, the burettes should be used just as ordinary burettes are used in the drop system.

[5] Our burettes are made by the Meyer Camera and Instrument Company, 31–33 East 27th Street, New York.

Digitized by Google

described above except that the counting of the drops is omitted.

The sugar titration described above has been adapted to the use of test-tubes instead of evaporating dishes, beakers, or flasks, and it has been adapted to the use of undiluted urine. The objection to the traditional dilution of urine to a sugar concentration of 0.5 to 1 per cent is that it cannot be properly made except on the basis of a preliminary sugar titration. (In the hands of medical students with a limited supply of measuring utensils the most frequent cause of gross errors in sugar determinations is one or another error or carelessness in connection with the dilution.) Our sugar titration can, of course, also be made with sugar solutions containing only one-half of 1 per cent of sugar and can therefore be made with sugar urines diluted to such a sugar concentration. It can also be adapted to the use of flasks instead of test-tubes, but only at the expense of chemicals. For those who prefer to use flasks, we recommend that the standard copper solution contain 3 per cent instead of 6 per cent copper sulfate. For 10 cc. of this solution (corresponding to 25 mg. of dextrose) 10 gm. of the dry alkaline salt mixture should be taken. If much less salt than that prescribed is used the white cuprous sulfocyanide is hydrolyzed and traces of red cuprous oxide begin to appear and the end-point of the titration becomes less readily visible. We believe that very few who have once become familiar with the use of test-tubes in sugar titrations will go back to the use of flasks, beakers, or other vessels.

Sugar in Urine.

No. of urine.	New method.	Benedict's method.	By the polariscope.
	per cent	*per cent*	*per cent*
1	1.8	1.78	1.81
2	4.16	4.2	4.12
3	2.94	2.94	2.99
4	1.41	1.43	1.39
5	0.98	1.05	0.98
6	6.66	6.56	6.63
7	1.56	1.54	1.52
8	2.84	2.96	2.89
9	4.5	4.56	4.45
10	3.62	3.74	3.68

Digitized by Google

Sugar urines frequently contain albumin. Albumin does not destroy the accuracy of the sugar titration described in this paper. Because of the foaming produced by albumin it is, however, desirable, indeed necessary, to use rather large test-tubes and to boil cautiously so that the contents do not boil over. The albumin does alter the appearance and probably also the composition of the cuprous precipitate, making it more flocculent and bulky; but, as already stated, the end-point of the titration is not changed or obscured by the presence of albumin.

Digitized by Google

Digitized by Google

THE DETERMINATION OF LACTOSE IN MILK.

By OTTO FOLIN and W. DENIS.

(*From the Biochemical Laboratories of Harvard Medical School and of the Massachusetts General Hospital, Boston.*)

(Received for publication, January 25, 1918.)

In this paper we describe two methods for the determination of the sugar in milk—a titration method and a colorimetric method. Of the two procedures we believe the titration to be the more accurate. The colorimetric method has, however, the advantage that by means of it a very large number of determinations can be made more or less simultaneously.

I. Titration Method.

In the sugar titration method of Folin and McEllroy 40.4 mg. of anhydrous lactose have the same reducing value as 25 mg. of dextrose. The lactose value was determined by us because it was thought that the method might prove quite as serviceable for the titration of the lactose of milk as for the titration of the dextrose of urine. Since albumin does not interfere with that titration it was hoped that the lactose of milk could be titrated directly, that is without any preliminary preparation of protein-free filtrates. A remarkably simple method for the determination of the sugar in milk would thus be secured. Our conjecture turned out to be correct, for we have encountered no difficulties in titrating the lactose of milk without first removing the protein and the fat. By means of large numbers of check determinations, using protein-free filtrates and determining the sugar by titration, by the polariscope, as well as colorimetrically, we have satisfied ourselves that the preliminary removal of the protein materials is superfluous. Omitting the preparation of protein-free milk filtrates eliminates not only the most tedious feature of the older methods for titrating the sugar in milk, but it also does away

Digitized by Google

with the cumbersome "corrections" for the volume occupied by the protein-fat precipitates.

Because of the extreme variations in the sugar content of urine it seemed desirable to be able to titrate it without preliminary dilution. In the case of milk the circumstances are entirely different, for the variations in the sugar content of this secretion are relatively very small, and a *suitable* preliminary dilution, without any uncertainty and without any approximate preliminary sugar titration, can be made. It has therefore seemed to us rather better to dilute milk before titrating, though the titration can be made quite well without any such dilution. We recommend a dilution of 1:4 (25 cc. in a 100 cc. flask) for cow's milk and 1:5 (5 cc. in a 25 cc. flask) for mother's milk. In emergencies, as when the quantity of human milk available for the sugar titration is very small, a dilution of 1:6 (2 cc. added to 10 cc. of water) can be used.

The titration is made as follows: Into a large test-tube introduce 2.8 to 3.4 cc. of the diluted milk (that is, nearly the full amount expected), 5 cc. of a 6 per cent copper sulfate solution, a pebble (to prevent bumping), and 4 to 5 gm. of a dry salt mixture (made by mixing, in powdered form, 100 gm. of disodium phosphate, 60 gm. of anhydrous sodium carbonate, and 30 gm. of sodium or potassium sulfocyanate). Shake, and boil gently for 4 minutes before adding any more milk. At the end of this time add more milk (0.02 cc. to 0.10 cc., depending on the amount of blue color remaining) and boil again. After each addition of milk (except the first) boil 1 minute. The total boiling period should be 5 to 7 minutes.

For the measurement of the milk we recommend the 5 cc. burette and other accessories, mentioned in the preceding paper in connection with the titration of sugar urines.[1]

Calculation: 4.04 multiplied by the degree of dilution (4, 5, or 6) and divided by the titration figure, gives the per cent of lactose present.

[1] We have up to date made over 200 sugar determinations by this new titration method—most of them on human milk. This work has developed into a relatively extensive study of the sugar content of human milk and will be published later.

Digitized by Google

II. Colorimetric Method for the Determination of Lactose.

Dehn and Hartman[2] have already described a colorimetric picrate method for the determination of the sugar in milk. Our procedure is materially simpler, and it gives, we believe, as accurate results as it is possible to obtain by a colorimetric picrate method.

The method is as follows: With an accurate 2 cc. pipette transfer 2 cc. of milk to a 100 cc. volumetric flask previously half filled with saturated picric acid solution. Fill to the mark with saturated picric acid solution, shake, and filter. Transfer 5 cc. of the filtrate to a large test-tube or 100 cc. volumetric flask, add 15 cc. of saturated picric acid solution and 3 cc. of 20 per cent sodium carbonate solution. Mix and place in a boiling water bath for 15 minutes. Cool and dilute to a volume of 100 cc. The standard consists of a solution of lactose in saturated picric acid solution. For use with cow's milk this solution should contain 5 mg. of lactose in 20 cc. For use with human milk it is necessary to employ two standards; namely, one containing 5 and the other containing 7 mg. of lactose in 20 cc. To 20 cc. of the standard add 3 cc. of the sodium carbonate solution and heat simultaneously with the milk filtrate. Cool, dilute to 100 cc., and compare the colors in the usual manner in a suitable colorimeter.

According to our experience accurate results cannot be uniformly obtained except by heating the standard simultaneously with the unknown and it is therefore absolutely necessary to prepare a new standard for each set of determinations. Nor can the color values of the standard and the unknown be more than 20 per cent apart.

In the following table are given a few figures representing parallel determinations of lactose in milk made by the colorimetric and by the titration methods.

[2] Dehn, W., and Hartman, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 404.

Digitized by Google

Determinations of Lactose in Cow's and in Women's Milk.

No.	Lactose.	
	Colorimetric method.	Titration method.
	per cent	*per cent*
Cow 57	4.42	4.41
" 41	4.08	4.30
" 37	4.13	4.22
" 22	4.54	4.46
" 39	4.87	4.84
" 45	4.51	4.54
" 31	4.21	4.23
" 31	4.37	4.45
" 44	4.60	4.50
" 38	4.64	4.68
Mother 1	3.08	3.10
" 8	5.73	5.75
" 9	5.07	5.05
" 10	4.79	4.83
" 12	6.73	6.63
" 13	6.64	6.53
" 16	5.87	5.87
" 17	6.37	6.40

Digitized by Google

NITROGEN CONTENT OF BACTERIAL CELLS.

I. METHOD.

By HAROLD C. BRADLEY and M. STARR NICHOLS.

(*From the Laboratory of Physiological Chemistry and the State Laboratory of Hygiene, University of Wisconsin, Madison.*)

(Received for publication, January 5, 1918.)

The production of acids, gases, and intermediate products, of toxins, agglutinins, and many other protein complexes serves to differentiate strains and groups of organisms and at the same time furnishes us with considerable interesting data in regard to bacterial metabolism. On the other hand, we have very little information as to the proximate and ultimate composition of bacteria. The difficulties attending the growth of the organism in sufficient quantity for accurate analysis and the difficulties of the analytical technique have largely prevented the development of this aspect of the work.

We know that higher plants differ from each other in their nitrogen content, and in the compounds characteristic of each. Enough work has already been done to indicate that wide differences may exist in the nitrogen content of bacteria. Nicolle and Alilaire (1) and Wheeler (2) give the results tabulated below for the nitrogen content of various organisms.

The results show the wide variation in the nitrogen content of various organisms and also the great discrepancies between analyses of the same organism by different persons. These results are of doubtful value, probably because of the variety of conditions maintained during growth, and because the technique allows errors of considerable magnitude where such small amounts of material were to be used. It was decided to apply the Folin method (3) to our problem and after certain adaptations we arrived at the following method for determining the nitrogen content of bacteria.

Digitized by Google

Kind of organism.	Nitrogen.	
	Nicolle and Alilaire.	Wheeler.
	per cent	*per cent*
B. proteus vulgaris	10.73	6.791
Typhoid	8.28	11.55
Anthrax	9.22	10.28
Colon	10.32	10.655
B. pyocyaneus	9.79	10.843
" *subtilis*	—	5.964
" *violaceus*	—	11.765
" *tuberculosis* "air-dried"	—	9.27
Chlorella vulgaris	3.96	—
B. dysenteriæ liquefaciens	8.89	—
" *pneumoniæ*	8.33	—
" *psittacosis*	9.55	—
Glanders	10.47	—
Sarcina aurantiaca	—	11.46

Method.

A battery of ten hard glass test-tubes was set up together with a sand bath and Bunsen burners to furnish the required temperature. Inverted funnels with bent stems connected with rubber tubes to a suction bottle were placed over the tubes to remove the fumes and exclude the dust.

The bacteria used in the experiment were cultures of *Bacillus diphtheriæ* which had been isolated from the throats of persons suffering from the disease. These bacteria were grown on Loeffler's blood serum medium slants for 72 hours. It usually required eight of these tubes to furnish enough material for one determination. The growth of organisms was removed without disturbing the solid medium, by a glass spade with rounded edges, made by flattening a small glass rod. The material was spread on a tared cover-slip and dried in a calcium chloride desiccator at 37°C. for 72 hours.

On account of the small amount of the material obtainable from a reasonable number of tubes, the weighing was done on an assay balance which was sensitive to 0.000005 gm. The weighing glasses were the thin, 15 mm. square cover-slips used in bacteriological work. They were cleaned in concentrated

Digitized by Google

nitric acid, washed in distilled water several times, and then finally in alcohol, out of which they were wiped dry when used.

Into each of the ten tubes was placed the following charge:

	gm.
Potassium sulfate	2.0
Copper sulfate	0.2
Sulfuric acid, concentrated	5.0

Weighed cover-slip and material.

The digestion was continued for 15 minutes after the liquid had become colorless. In practically all the material digested, there was noticed a small quantity of material which digested with difficulty. After the digest had cooled somewhat it was diluted with water, made alkaline with sodium hydroxide, and

TABLE I.

Cover-glass and dry bacteria.	Dry bacteria.	Net weight of nitrogen.	Nitrogen.
gm.	*gm.*	*gm.*	*per cent*
0.080280	0.004655	0.000370	7.9
0.081060	0.004670	0.000390	8.3
0.076380	0.005730	0.000485	8.5
0.076925	0.007095	0.000575	8.1
0.085820	0.003360	0.000290	8.6
0.070600	0.005420	0.000440	8.2
0.085050	0.007300	0.000581	8.0
0.058450	0.004040	0.000357	8.8
0.056470	0.002880	0.000245	8.9
0.079020	0.004810	0.000413	8.7
0.073330	0.001485	0.000120	8.4
0.080460	0.002575	0.000250	9.7*
0.097390	0.003390	0.000291	8.6
0.089075	0.001350	0.000154	11.4*
0.065480	0.006170	0.000470	7.6
0.068180	0.004625	0.000490	10.5*

* On account of the great variance from the average it seems permissible to omit these results from the average.

Digitized by Google

aspirated with ammonia-free air into 0.1 N sulfuric acid. The acid solution containing the ammonium sulfate was Nesslerized and compared with a standard solution of ammonium sulfate similarly Nesslerized, by means of the Duboscq colorimeter. A convenient strength of standard to use, if the quantity digested is about 5 mg., contains an ammonia equivalent of 0.0000025 gm. of nitrogen per cc. With every set of determinations two blanks were run and the average of these was subtracted from the nitrogen found.

The results of sixteen separate determinations are given in Table I.

A nitrogen determination was made on the Hoffmann bacillus, a diphtheroid organism, by this method with the results as recorded in Table II.

TABLE II.

Nitrogen Content of Bacillus hoffmanni.

Cover-glass and dry bacteria.	Dry bacteria.	Net weight of nitrogen.	Nitrogen.
gm.	*gm.*	*gm.*	*per cent*
0.074110	0.002900	0.000277	9.6
0.075590	0.003730	0.000356	9.5
0.100210	0.006235	0.000600	9.6
0.071810	0.009890	0.001020	10.3

In order to determine the accuracy of the method, a macro-Kjeldahl determination was made on a larger quantity of the dried diphtheria bacteria with the following results:

No. 1..... 7.9 per cent nitrogen.
" 2..... 8.1 " " "

SUMMARY.

In summing up the results of these determinations the following points seem important:

1. The nitrogen content of *Bacillus diphtheriæ* is

	per cent
Regular Kjeldahl method	8.0
Micro-Kjeldahl method	8.35

Digitized by Google

The nitrogen content of *Bacillus hoffmanni* is found to be 9.75 per cent by this method. Under like cultural conditions then, these two organisms show marked differences in this particular.

2. The sources of error met when an analysis is made are overcome as follows: (*a*) The possibility of contamination of the material with small pieces of medium is eliminated by the use of a molded glass spade which rubs off the growth without scratching the medium. (*b*) The presence of moisture in the organism when weighed. The organisms were dried under like conditions which were purely arbitrary but which were found to be adequate for reducing the film to constant weight. The analytical results themselves are sufficiently alike to show that the moisture content must have been uniform for each type. (*c*) The error in weighing. That this error is only apparent is seen by comparing the accuracy of the scale used with the amount weighed. (*d*) The error in ammonia obtained from reagents. This was entirely accounted for by running measured blanks with every determination. (*e*) Loss of ammonia by aspiration. In the first few practice runs there was experienced a noticeable error at this point; however, the rate of aspiration at the start was reduced and the total length of time prolonged which overcame this source of error.

CONCLUSIONS.

It is possible to determine the nitrogen content of any bacterium which will grow on a solid medium without liquefaction of that medium, by this method, provided as much material as 5 mg. can be obtained.

It is our intention to study further the nitrogen differences in bacteria of different groups and of different members of the same group.

BIBLIOGRAPHY.

1. Nicolle, M., and Alilaire, E., Note sur la production en grand des corps bactériens et sur leur composition chimique, *Ann. Inst. Pasteur*, 1909, xxiii, 547.
2. Wheeler, in Vaughan, V. C., Vaughan, V. C., Jr., and Vaughan, J. W., Protein Split Products in Relation to Immunity and Disease, Philadelphia and New York, 1913, 65.
3. Folin, O., and Farmer, C. J., A new method for the determination of total nitrogen in urine, *J. Biol. Chem.*, 1912, xi, 493.

Digitized by Google

Digitized by Google

IONIZATION OF PROTEINS AND ANTAGONISTIC SALT ACTION.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, January 30, 1918.)

I.

In 1899 Wolfgang Pauli[1] and the writer[2] independently reached the conclusion that electrolytes when acting on proteins formed ion-protein compounds. The writer anticipated that these ion-proteins would explain the mystery of many life phenomena. He was especially interested in one of the most universal physiological actions of salts; namely, the antagonistic salt action, for which the annihilation of the effects of a high concentration of a salt with univalent cation, *e.g.* NaCl, by a low concentration of a salt with bivalent cation, *e.g.* $CaCl_2$, is perhaps the best known example. Although he and many others tried to demonstrate this type of antagonism in proteins they never succeeded. It was a further disappointing fact that Hardy[3] found that globulins apparently form electrically neutral compounds with neutral salts and this seemed to harmonize with the older observations of Liebermann and Bugarszky. Pauli[4] had expressed the idea that a low concentration of salts ionizes globulins and thereby causes their solution, but even he assumed not a real chemical combination but adsorption between the globulin and the salt.

Meanwhile, many workers, and especially Pauli and his pupils, had developed a number of methods for discriminating between the chemical behavior of ionized and non-ionized proteins, but

[1] Pauli, W., *Arch. ges. Physiol.*, 1899, lxxviii, 314.

[2] Loeb, J., *Arch. ges. Physiol.*, 1899, lxxv, 303; *Am. J. Physiol.*, 1900, iii, 327.

[3] Hardy, W. B., *J. Physiol.*, 1905–06, xxxiii, 251.

[4] Pauli, *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

Digitized by Google

the surest method of producing ionized proteins was to treat proteins with either acid or base. It was generally found that when a protein with little tendency to electrolytic dissociation is treated with acid of the right concentration a protein salt is formed which undergoes a strong electrolytic dissociation, while the addition of a neutral salt diminishes this degree of dissociation, as was to be expected. Loeb and Wasteneys,[5] and later the writer alone,[6] were able to show that the toxic effect of acid can be diminished or suppressed by the addition of neutral salts. This was the first direct support of the idea that antagonistic salt action was due to a transformation of ionized protein into electrically neutral protein.

The second case where it seemed possible to attribute antagonistic salt action to a transformation of ionized into non-ionized protein consisted in the observation that the diffusion of a low concentration of KCl into the egg of *Fundulus* is impossible or extremely slow when this salt is alone in solution, but is rendered possible through the addition of a small amount of a second salt, *e.g.* NaCl ("salt effect"), and that the diffusion of KCl is rendered impossible again when more of the second salt is added. The analogy with the solubility of globulin suggested that the addition of a small quantity of the second salt caused an ionization of some protein in the membrane of the egg which was suppressed again by the addition of more salt; and the writer expressed this idea in a preliminary notice.[7] But this suggestion faces the uncomfortable fact already referred to that according to Hardy globulins form non-ionized molecules with neutral salts.[3]

The writer, however, has found a method which suggests strongly that the antagonism between NaCl and $CaCl_2$ is due—at least in the case to be discussed—to the fact that an ionization of protein is caused by NaCl and that this ionization or its effect upon the swelling is suppressed by a comparatively small quantity of $CaCl_2$.

[5] Loeb, J., and Wasteneys, H., *Biochem. Z.*, 1911, xxxiii, 489; 1912, xxxix, 167.

[6] Loeb, *J. Biol. Chem.*, 1915, xxiii, 139; 1917, xxxii, 147.

[7] Loeb, *Proc. Nat. Acad. Sc.*, 1916, ii, 511.

Digitized by Google

Some of the experiments have already been published.[8] Into a cylindrical funnel 2 gm. of finely powdered *non-bleached*[9] Cooper's gelatin are put; the powder is held in the cylinder by a circular piece of filter paper. Three such funnels each with 2 gm. of gelatin are prepared. The one (I) is perfused six times in succession with 25 cc. of distilled water; the second (II) is perfused twice with 25 cc. M/8 NaCl and then four times with 25 cc. of distilled water; the third (III) is perfused six times with 25 cc. M/8 NaCl. In I and III a moderate swelling occurs, which soon reaches its maximum. The gelatin in II, first treated with NaCl and subsequently with distilled water, swells several hundred per cent more than either the gelatin treated only with distilled water (I) or the gelatin (III) treated only with NaCl.

The explanation of this experiment is as follows. Gelatin II forms under the influence of a comparatively high concentration of NaCl (M/8 or M/4) a compound with NaCl which is capable of ionization. This ionization is lowered through the presence of the highly concentrated NaCl solution. When, however, this latter solution is washed away by successive perfusions of the gelatin with distilled water the gelatin-NaCl compound can dissociate into gelatin and an inorganic ion, the nature of which we shall discuss later. It has been shown by Pauli and by Procter[10] that the swelling of protein under the influence of acid or base is due to the ionization of protein by the acid and the writer assumes that the increase in the amount in swelling of the mass of gelatin first treated with NaCl and then washed with distilled water is due to the fact that part of the gelatin is transformed into protein ions by the salt, and that this ionization and the swelling can only appear when the NaCl solution held in the capillary spaces of the powder is washed away. This statement is supported by the following facts.

1. A large number of funnels are prepared, each with 2 gm. of powdered gelatin and each is perfused twice with 25 cc. M/8 NaCl. Subsequently each funnel is perfused three times with

[8] Loeb, *J. Biol. Chem.*, 1917, xxxi, 343.

[9] Bleached gelatin as well as bleached pig's bladder does not give the same results, probably on account of an alteration in the constitution of the protein.

[10] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

Digitized by Google

25 cc. of a definite NaCl solution lower than M/8 NaCl; namely, M/16, M/32, M/64, M/128, etc., down to M/2048 or lower. It is found that after three perfusions the additional swelling of the gelatin becomes noticeable in the funnel which has been washed with M/64 or less concentrated NaCl solution and that the additional swelling increases rapidly with the diminution of the concentration of the NaCl solution. The explanation is that concentrations of NaCl above M/64 suppress the electrolytic dissociation of the gelatin-NaCl compound to such an extent that the swelling will not exceed that caused when the gelatin is perfused permanently with M/8 NaCl. Concentrations of M/64 and below no longer suppress the ionization of the gelatin salt completely and the suppression is the smaller the greater the dilution of the NaCl used for perfusion. Hence the swelling of the gelatin increases rapidly with the dilution of the NaCl solution below M/64 and reaches a maximum when distilled water is used for the perfusion. Table II gives adequate examples. This fact had already been published.[8]

2. The second proof consists of the fact that when the gelatin powder is first perfused with M/8 NaCl and then with a solution of a non-electrolyte, the increasing dilution of the non-electrolyte is without effect. Table I illustrates this statement. The table gives the result of four sets of experiments. In each set about fourteen cylindrical funnels contained 2 gm. of powdered gelatin and each funnel was perfused twice with 25 cc. M/8 NaCl. After this the various funnels of one series were perfused with solutions of either NaCl or glycerol or cane sugar or ethyl alcohol of increasing dilutions. The swelling was measured after three perfusions. In order to allow all the liquid not held by the gelatin (or by adhesion) to filter out the final measurement was taken after 24 hours. Precautions were taken to avoid error by evaporation. Since the cross-section of all the cylindrical funnels was the same the increase in the height of the cylindrical mass of gelatin above the height of the mass reached by the perfusion with M/8 NaCl may serve as a measure of the additional swelling.

The difference between the influence of the electrolyte and the non-electrolyte is very striking. Gelatin treated with M/8 NaCl does not show any further swelling when treated with M/8 or M/16 NaCl; from M/64 on the swelling begins, gradually increas-

Digitized by Google

ing and reaching its maximum at M/1024. Gelatin treated with M/8 NaCl and then treated with solutions of non-electrolytes from 2 M down swells to the same extent as if it were treated with distilled water. This harmonizes with the assumption that the swelling is determined by the degree of electrolytic dissociation of the gelatin.

3. It has been shown by Pauli and his fellow workers that ionized protein can no longer be precipitated by alcohol.[4] If the additional swelling of gelatin in distilled water caused by the previous treatment with M/8 NaCl was due to an increase in ionization such gelatin should resist precipitation by alcohol. This is most strikingly the case. When we dissolve the three kinds of

TABLE I.

	Increase in swelling of powdered gelatin (which had first been perfused with 50 cc. M/8 NaCl) after three perfusions with 25 cc. of													
	4 M	2 M	M	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	H_2O
NaCl.....						0	0	2.5	6	15	21	33.5	42	
Glycerol.....	34	37	40	40	40	43	42	42	40	41	41	41	43	42
Cane sugar.....		35	41	49	49	51	51	50	50	51	51	51		48
Ethyl alcohol..	42.5	48.5	54	48.5	48.5	48.5	49.5	49.5	49.5	47	47.5	47		50.5

powdered gelatin, I treated with water alone, II treated first with M/8 NaCl and then with water, and III treated with salt (M/8 NaCl) alone, and measure the quantity of alcohol needed to precipitate 5 cc. of a 3 per cent gelatin solution of each of the three samples, we shall find that it requires about 5 cc. of 95 per cent alcohol to cause a heavy precipitate in I, and about 7 cc. to cause the same precipitate in III; while in II no precipitate is formed even by the addition of 20 cc. or more of alcohol; the solution assumes only a bluish tint upon the addition of about 20 cc. of alcohol.

4. It was shown in the previous paper that any neutral salt with univalent cation acts like NaCl upon powdered gelatin, inasmuch as a perfusion of gelatin with a neutral salt of Li, K, or NH_4, when followed by a perfusion with distilled water, causes an additional excessive swelling; while the perfusion of the powdered gelatin with neutral salts of the bivalent cations, Mg, Ca, Sr, and Ba, does not cause any additional swelling when the

Digitized by Google

gelatin is afterward perfused several times with distilled water. It would, therefore, follow that gelatin treated by neutral salts with bivalent cations increases its mass of gelatin ions either not at all or only to a negligible extent. This is apparently the case. When 2 gm. of powdered gelatin are first perfused twice with M/8 $CaCl_2$ and are then perfused three times with 25 cc. of distilled water, and if then a 3 per cent gelatin solution is prepared, 5 cc. of such a solution are precipitated by slightly less than 5 cc. of alcohol (at room temperature), which is approximately the same figure as that for gelatin not treated with salt. The gelatin is, therefore, apparently not ionized by Ca.

5. This difference in the action of neutral salts with bivalent and univalent cations, namely, that the latter ionize the gelatin while the bivalent cations will apparently not form ionizable compounds, is the basis of the antagonistic action between the two types of salt. When we treat powdered gelatin with a mixture of NaCl and $CaCl_2$ the addition of a comparatively small amount of $CaCl_2$ to M/8 NaCl will inhibit the swelling which would follow if the gelatin were perfused first by the NaCl alone and then by distilled water. It can be shown that when we perfuse 2 gm. of powdered gelatin twice with 25 cc. of 50 cc. M/8 NaCl + 4 cc. M/8 $CaCl_2$, and then three times with distilled water, the gelatin is precipitable by alcohol; while if we use the pure M/8 NaCl or 50 cc. M/8 NaCl + 1 or 2 cc. of M/8 $CaCl_2$ the 3 per cent gelatin solution freed from the salt is not precipitable by alcohol. We, therefore, come to the conclusion that the phenomenon of swelling of gelatin under the influence of neutral salts with univalent cation and the inhibition of this swelling by neutral salts with bivalent cation (alkaline earths) are due to the fact that the former salts cause the formation of ionizable gelatin salts, while the latter apparently cause the formation of non-ionizable gelatin salts.

II.

We may raise the question: Which of the possible cases of ionization actually occurs when gelatin is treated with neutral salts with univalent cation, like NaCl? If we assume that the gelatin molecule combines with both anion and cation the neutral complex gelatin $\begin{matrix} \diagup \mathrm{Na} \\ \diagdown \mathrm{Cl} \end{matrix}$ can give rise to three types of protein ions:

Digitized by Google

$$(1)\quad \text{Gelatin}^{-}\text{-Cl} + Na^{+}$$
$$(2)\quad \text{Gelatin}^{+}\text{-Na} + Cl^{-}$$
$$(3)\quad \text{Gelatin}^{+-} + Na^{+} + Cl^{-}$$

In (1) gelatin^{-} ion would be negative, in (2) gelatin^{+} ion would be positive, and in (3) the gelatin ion would have both a negative and a positive charge in different parts of the molecule. We shall see that the facts speak in favor of (1); namely, an exclusive or a prevailing formation of negative gelatin ions. According to our assumption the additional swelling of gelatin due to ionization should begin when the salt solution used for perfusion to wash out the M/8 NaCl is so dilute as not to suppress the electrolytic dissociation of the gelatin-salt compound completely. Since this suppression depends upon the common ion of the gelatin-salt compound and the salt, it should be possible to ascertain which this common ion is, the anion or the cation of the salt. If, for instance, the dissociation of the gelatin salt occurs according to (1) into gelatin^{-} + Na^{+}, the lowest concentration of Na_2SO_4 which just allows the additional swelling of a gelatin previously treated with M/8 NaCl should be about one-half of that in which the swelling begins when lower concentrations of NaCl are used; since, *e.g.*, a M/256 Na_2SO_4 solution sends about twice as many Na ions into solution as does a M/256 NaCl solution. In other words, that concentration of a salt which is just able to inhibit completely the subsequent swelling of gelatin previously treated with M/8 NaCl should depend only upon the concentration of the cation of this salt, if the type (1) of the dissociation of the gelatin salt exists; namely,

$$(1)\quad \text{Na-gelatin} \rightarrow \text{gelatin}^{-} + Na^{+}$$

This is the case. We know from Table I that the inhibiting concentration of NaCl for the additional swelling[11] of gelatin previously treated with M/8 NaCl is M/64 NaCl. In this concen-

[11] By additional swelling we mean the swelling which takes place as a consequence of the previous treatment with M/8 NaCl, when the excess of the salt is removed and the ionization of the gelatin salt no longer too much depressed.

Digitized by Google

tration an increase of 5 mm. in the height of the cylindrical mass of gelatin will show itself. With a further dilution of NaCl the swelling of the gelatin increases very rapidly.

We now undertook the following series of experiments. We perfused 2 gm. of powdered non-bleached gelatin, as in the previous experiments, first with 50 cc. M/8 NaCl, and followed this with a perfusion with solutions of different neutral salts of increasing dilution. Table II gives the results of such a series of experiments.

TABLE II.

	Additional swelling of 2 gm. powdered gelatin previously perfused twice with 25 cc. M/8 NaCl and three times with 25 cc. of the following solutions.											
	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
NaCl	0	0	2.5	6	15	21	33.5	42	48			54
NaBr	0	2.0	2.0	5	12.5	17.5	27	43	42	46	53	52.5
$NaNO_3$	1	?	4	7	12.5	19.5	29.5	39	43.5	51	49	54
Na acetate	1.5	1.5	3	6.5	15	22	31	38	45.5	44	48	55
NaCNS		3.5	4	7	12	21	31	40	46	53	58	60
Na_2SO_4	1	1.5	2.5	3.5	8	14	22.5	30	39	47	55	54
Na_2 oxalate	2	2	2	3.5	6.5	14.5	24	29	35	46	50	56
Na_2 tartrate	2	2	2	3	5.5	13.5	23	32.5	43	46	52.5	56.5
Na_2 malate	0	1	1	2.5	5	13	20	31	35	39	43	51

While the sodium salts with monovalent anion all show the beginning of additional swelling (*i.e.* swelling amounting at least to 5 mm. increase in height of column) in a dilution of M/64 the sodium salts with bivalent anion show this increase in twice this dilution; namely, M/128. This would indicate that the metal ion alone or prevailingly determines the degree of electrolytic dissociation of the gelatin sodium salt. In other words, the gelatin salt formed, when gelatin is treated with NaCl, dissociates exclusively or prevailingly in the form gelatin $^-$ + $Na.^+$ The anion plays obviously a minor if not a negligible part.

When we test 3 per cent solutions of such gelatin with alcohol we find that the solution always becomes non-precipitable as soon as the swelling becomes marked.

Digitized by Google

When we treat gelatin first with M/8 NaCl and then with dilute solutions of salts of other monovalent cations the results should be essentially the same as if we treated the gelatin with solutions of NaCl, since a compound gelatin-Na should exchange its metal with Li or K or NH_4 without essential molecular alteration. Hence the results should remain practically the same if a treatment with M/8 NaCl is followed by solutions of NaCl or of LiCl or KCl. This is true, as Table III shows.

TABLE III.

	Additional swelling of powdered gelatin perfused twice with 25 cc. M/8 NaCl and three times with 25 cc. of the following solutions.											
	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
LiCl	1	1.5	3.5	6	10.5	15	24.5	34.5	44.5	52	53.5	57.5
KCl	1	2	2.5	6.5	11.5	16	24	33	38.5	44	46.5	53.5
NH_4Cl	2	2	2	5	13	23	22.5	39.5	48	47		56
Li_2SO_4	3	0	2	2.5	3.0	10	19.5	30.5	45.5	51	51	60
K_2SO_4	1	1.5	0.5	3.0	6.5	11	20	29	40.5	42	50.5	54.5
$(NH_4)_2SO_4$	1.5	1.5	2	3	6	12	18	26	42	50	45	53
K_2 oxalate	1	1	1.5	3.5	7.5	17	22.5	25	33.5	43	46.5	55
$(NH_4)_2$ oxalate	0	1	1	2.5	5	11.5	22.5	27	36.5	48.5	49	54
K_2 tartrate	0	0	1.5	1.5	7	13	19	28	37	42	46	53
$(NH_4)_2$ tartrate	2	1.5	2	5 ?	7	16.5	22	35.5	43.5	49.5	52	55

For the chlorides the swelling becomes noticeable at a dilution of M/64, for the sulfates, oxalates, and tartrates at nearly twice the dilution; namely, about M/128. Only Li_2SO_4 is an apparent exception for reasons still to be investigated.

It was shown by special controls that if we first treat powdered gelatin with M/8 Na_2SO_4 and follow this by weaker concentrations of Na_2SO_4 we get the same critical value for the commencement of swelling as when we first treat the gelatin with M/8 NaCl and follow this with weaker concentrations of Na_2SO_4; namely, M/128. M/8 LiCl followed by different concentrations of LiCl gives the same critical value for the commencement of swelling as M/8 NaCl followed by different concentrations of LiCl.

If the anion has a distinct effect it must show itself in com-

Digitized by Google

paring the effects of washing with $MgCl_2$ and $MgSO_4$ and of $CaCl_2$ and $CaSO_4$. When we perfuse powdered gelatin first with M/8 NaCl and then with $CaCl_2$ solutions of a higher degree of dilution we must expect a lower limit of concentration where the swelling begins, since $CaCl_2$ by replacing the NaCl in combination with gelatin transforms the ionizable gelatin into a less ionizable or otherwise modified complex. The concentration of $CaCl_2$ must therefore become comparatively low before swelling becomes possible. If the additional swelling, *i.e.* the ionization of the gelatin, depends on the cation alone or predominantly, $CaSO_4$ and $MgSO_4$ should have the same critical concentration for the commencement of the swelling, which is the case, as Table IV shows. The critical concentration for all the salts is M/512.

TABLE IV.

	Additional swelling of powdered gelatin perfused twice with 25 cc. M/8 NaCl and then three times with 25 cc. of the following solutions.											
	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	M/4096	M/8192	H_2O
$MgCl_2$	0	0	0	0	0	4	13	26	42	49	53	52
$MgSO_4$	1.5	0	0	−1	0	1	7.5	22.5	38	48	55	63
$CaCl_2$	0	0	0	0	0	1.5	8	24	39	48	51	47
$CaSO_4$					−3.5	−2.5	+8.5	18.5	37.5	43	47	55
$SrCl_2$	0	0	0	0	0	1	8	18	36	43	52	52
$BaCl_2$	0	0	0	0	0	1	10	21	40	45	51	50

All these facts support the idea that neutral salts form compounds with gelatin which dissociate into a positive metal ion (that of the salt used) and into a negatively charged protein ion which may or may not contain the anion of the salt. This idea was tested by experiments on the migration, in an electric field, of gelatin treated with M/8 NaCl and then freed from the salt by washing. Such gelatin migrates to the anode as our theory demands.

III.

The idea that only the cation enters into the ionization of gelatin might be interpreted to mean that a reaction of the following kind occurs between gelatin and NaCl:

Digitized by Google

$$RCOOH + NaCl \rightarrow RCOO\text{–}Na + HCl.$$

In this case the supernatant solution should have an acid reaction. This was not the case.

Pauli makes another suggestion based on the fact that proteins in general are stronger acids than bases, and that hence they must undergo a stronger hydrolytic dissociation where they act as base than where they act as acid. If such a protein combines with NaCl the Cl would undergo hydrolytic dissociation and be washed away as free HCl. But if this were the case, the water with which NaCl-gelatin is washed should have an acid reaction. Although we were not able to demonstrate such an acid formation, we should remember that little acid was formed at the utmost and that some of it may have been bound again by gelatin molecules. The important fact is that under the influence of a neutral salt, of the type NaCl, the gelatin forms a sodium gelatinate, which dissociates electrolytically into a negatively charged gelatin ion and a positively charged metal ion—that of the salt used.

This assumption is further supported by hydrogen ion determinations which Dr. Dernby has made in my laboratory and which I incorporate here with his permission. The writer had shown in a previous paper that pig's bladder behaves like powdered gelatin inasmuch as it shows a considerable additional swelling in H_2O when previously treated with NaCl or any neutral salt with univalent cation; while no such additional swelling in H_2O occurs after previous treatment with neutral salts with bivalent cation. Dr. Dernby found that when the membranes treated with M/8 NaCl (or KCl, etc.) were afterward put into distilled water the latter became slightly alkaline; but that this was not the case when the pig's bladder was previously treated with $CaCl_2$.

Dr. Dernby determined the hydrogen ion concentration by Sörensen's colorimetric method, using his standard phosphate solutions and neutral red as indicators. In all these experiments the hydrogen ion concentration changes in the same way as the swelling. Where a strong additional swelling occurs, as in H_2O after a previous treatment in NaCl, the hydrogen ion concentration diminishes; where no additional swelling occurs no change

Digitized by Google

in the hydrogen ion concentration is observed. In the M/8 NaCl solution where the membrane does not swell no change in the hydrogen ion concentration occurs.

Dr. Dernby's method was as follows. Pieces of pig's bladder of equal weight (about 0.75 gm.) were put into 50 cc. of a salt solution for 1 hour, then washed repeatedly, and transferred into 50 cc. of distilled water (except in the controls, where the bladder remained permanently in distilled water or in the salt solution).

Table V gives his results.

TABLE V.

After	Hydrogen ion concentration of a solution containing pig's bladder treated as stated (expressed with Sörensen's symbol pH).					
	Control in distilled water.	In M/8 not washed.	In M/8 NaCl washed in H_2O after 1 hr.	KCl	NH_4Cl	Na_2SO_4
hrs.						
0	About 6.4	6.4	6.4	6.2 to 6.4		
½	6.4	6.4	6.4			
1	6.4	6.4	6.4	Washed.		
2	7.0	6.4	7.1	6.8	6.8	6.9
3	7.1	6.6	7.2	7.0	7.0	7.0
5	7.1	6.6	7.3	7.3	7.3	7.3
24	7.1	6.9	7.4	7.3	7.3	7.3

In these experiments the distilled water and salt solutions contained CO_2 and this is the reason why at the first reading the H ion-concentration is so high (6.2 to 6.4 instead of 7). The determinations prove that the solutions become more alkaline when the pig's bladder was first treated with a neutral salt with monovalent cation. This agrees with our assumption of an electrolytic dissociation into a negative gelatin ion and a positive Na^+ ion, since slight hydrolytic dissociation would lead to the formation of a stronger base (NaOH) and a weaker acid, gelatin-COOH.

We had shown that the bivalent metals, Mg, Ca, Sr, and Ba, form in all probability less ionizable compounds with gelatin. Hence we should expect that a previous treatment of pig's blad-

Digitized by Google

der with Ca should not increase the alkalinity of the distilled water. This is actually found to be the case by Dr. Dernby (Table VI).

TABLE VI.

After	Hydrogen ion concentration of a solution containing pig's bladder treated as stated (expressed with Sörensen's symbol pH).			
	Control in distilled water.	In M/8 $CaCl_2$ 1 hr. then in distilled water.	M/8 $MgCl_2$	M/8 $SrCl_2$
hrs.				
0		6.4		
½		6.4		
1	About 6.5	6.4		
		Then transferred into distilled water; immediately after transferring the H ion concentration was in all cases the same pH = 6.2 to 6.4.		
3	6.7	6.4	6.5	6.5
5	7.0	6.6	6.7	6.7
24	7.1	6.9	6.9	6.9

It is obvious that pig's bladder previously treated with solutions of $MgCl_2$, $CaCl_2$, and $SrCl_2$ does not cause a change of the same order in the reaction of the water as was found for bladder treated with NaCl and NH_4Cl. This harmonizes with the assumption that the bivalent metals form non-dissociable or less dissociable gelatin salts.

IV.

In the previous note[8] the writer has stated that the additional swelling caused by a treatment with NaCl was observed in powdered gelatin and in pig's bladder, but not in solid blocks of gelatin. It was found that the cause of this difference is the fact that it is easy to ionize large masses of powdered gelatin with a salt and then wash the unnecessary salt solution away, while this is difficult if not impossible with solid blocks of gelatin. The salt solution apparently diffuses slowly in and out of the block. For this reason the following modification of the method was adopted. Powdered gelatin was treated with a M/8 NaCl solu-

Digitized by Google

tion in the usual way, and the salt solution was then removed by washing the powdered mass three or four times with distilled water. After all the water had run off the mass of gelatin was poured into a beaker and completely liquefied by heating for about 10 minutes in a water bath of about 40°. This homogeneous liquid mass of gelatin was then put into a bag of collodion and exposed to the draft of an electric fan for about 24 hours, when it had lost most of the water. When such blocks of gelatin, previously treated with M/8 NaCl and freed from the NaCl solution by repeated washings with H_2O, were put into H_2O they showed the same excessive swelling as had been observed in the case of the powdered gelatin; while this excessive swelling was prevented when the block of gelatin previously treated with M/8 NaCl was put back into M/8 NaCl.

The following is an example. Into each of three cylindrical funnels were put 2 gm. of finely powdered Cooper's unbleached gelatin. The gelatin in one funnel (I) was perfused six times in succession with distilled water. The second (II) was perfused twice with 25 cc. M/8 NaCl, then four times with 25 cc. of distilled water; the third (III) was perfused six times with 25 cc. M/8 NaCl. After all the water had run out, the weight of the first mass of gelatin was 34 gm., that of the second, 59.15 gm., and of the third 24.4 gm. The previous treatment with salt increased the swelling from 32 to 57 gm. Incidentally the reader will see that contrary to Hofmeister's statement the gelatin did not swell more when permanently treated with M/8 NaCl than it did when permanently treated with H_2O, since in the latter case it absorbed 32, in the former only 22 gm.—of which part was NaCl.[12] These three lots of gelatin were then put into collodion bags and exposed to the fan until their respective masses were 8.4 (I), 7.4 (II), and 6.05 (III) gm. The three masses of gelatin were then liquefied by heating to about 40°C., poured into a flat Petri dish, allowed to gelatinize, and then blocks of equal size were stamped out of each; the respective weights of the three blocks were 7.6 (I), 6.04 (II), and 4.19 (III) gm. I and II were put into distilled water, III was put into M/8 NaCl. After 24 hours the weighing gave the following result:

[12] Ash determination showed that the gelatin contained in this case the same proportion of NaCl and water as did the M/8 NaCl solution.

Digitized by Google

I	II	III
22.02 gm.	52.8 gm.	12.9 gm.

The gelatin treated first with M/8 NaCl swelled after removal of the salt two and one-half times as much in distilled water as did the gelatin not treated previously with NaCl. We are, therefore, justified in saying that blocks of gelatin behave in the same way as does powdered gelatin if only care is taken that the excess of salt is removed from the block before it is exposed to the distilled water. If a block of gelatin is first put into M/8 NaCl and then into distilled water, we get different results on account of the slowness of the diffusion of the salt into and out of the block.

In experiments with powdered gelatin we had found that the valence of the cation was much more effective than the valence of the anion. A previous treatment of powdered gelatin with M/8 $CaCl_2$ (or $MgCl_2$ or $SrCl_2$ or $BaCl_2$) did not cause the excessive swelling which was caused by M/8 NaCl, and it was found that the addition of a small amount of $CaCl_2$ to NaCl stopped the after-effect of NaCl upon the subsequent swelling of gelatin in H_2O (antagonistic salt action). The same is true for blocks of gelatin as the following experiment shows.

2 gm. of powdered gelatin were put into each of three cylindrical funnels. Funnel I was perfused six times with 25 cc. H_2O; funnel II twice with 25 cc. M/8 $CaCl_2$ and then four times with 25 cc. H_2O; and funnel III six times with 25 cc. M/8 $CaCl_2$. The next day (after all the water had drained off) the weight of the gelatin liquefied by heating to about 40° was

I	II	III
27.8 gm.	25.8 gm.	24.3 gm.

The $CaCl_2$ treatment had, therefore, little effect upon the swelling of powdered gelatin. The mass of the three lots was then reduced by evaporating in a collodion sac to 7.6, 7.1, and 7.3 gm. each and blocks of (I) 6.2, (II) 6.0, and (III) 4.7 gm. were cut out. Blocks I and II were put into distilled water, and block III into M/8 $CaCl_2$. After 41 hours the weight was as follows:

I	II	III
25.4 gm.	24.1 gm.	17.3 gm.

Digitized by Google

Comparing I and II we notice that the previous treatment with $CaCl_2$ did not increase the swelling of the block of gelatin.

In the same way all the experiments on antagonistic salt action described in the previous paper[8] could be repeated with solid blocks of gelatin.

These and other experiments allow us to state that the influence of salts upon the swelling of powdered gelatin published in the previous paper holds for solid blocks of gelatin also if only care is taken to remove the excess of salt from the block. The results obtained on powdered gelatin can, therefore, be utilized for the theory of swelling of gelatin in general.

V.

Our results contradict the conclusions which are drawn by many authors from the old experiments of Hofmeister[13] on the influence of neutral salts and sugar on the swelling of gelatin. Hofmeister stated that the salts, according to their effect upon the swelling of gelatin, may be divided into two groups. The one group makes the gelatin swell more than distilled water, the other makes it swell less than distilled water. The former group includes NaBr, $NaNO_3$, NH_4Cl, NaCl, KCl. The second group includes the acetates, citrates, tartrates, and sulfates; but also alcohol, grape sugar, and cane sugar. From these observations it has generally been concluded that the anion is the decisive factor for the swelling; and that Hofmeister's results agree with the influence of salts on other qualities of proteins. Such a conclusion overlooks Hofmeister's distinct statement that alcohol and sugars act like acetates and tartrates. This fact is of importance since it confirmed Hofmeister in his idea that the attraction of the salt for water was one of the factors by which salts and sugars influence the swelling of gelatin. Thus he states that the sulfates, tartrates, citrates, and acetates, attract water more powerfully than chlorides or bromides and hence prevent the gelatin from absorbing as much water as it does in pure water or in the presence of chlorides.

Hofmeister's method cannot give any clear idea concerning the influence of salts on the swelling of gelatin since this influence depends chiefly upon the ionization of the gelatin. Two proc-

[13] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 210.

Digitized by Google

esses are necessary to obtain the correct estimate of the effect of this ionization; first, treatment of the whole mass of gelatin with a sufficiently high concentration of the neutral salt (M/8 or M/4), and such a treatment can only be effective if the protein is in finely divided condition; and second, removal of the excess of salt in order to permit the electrolytic dissociation of the protein. Neither condition is fulfilled in Hofmeister's method. This also explains why he got similar results with sugar and alcohol as with sodium acetate and sodium sulfate. The differences Hofmeister observed in the action of different salts are comparatively slight and they cannot be used for a theory of the action of salts on swelling.

Lenk[14] tried to demonstrate the antagonistic action between NaCl and $CaCl_2$ on gelatin. He used Hofmeister's method, trying to show that blocks of gelatin swell less in NaCl solution when $CaCl_2$ is added, but the effects observed are small and, as the writer believes, within the limits of error of such experiments. Fenn[15] tried to show that gelatin solutions require less alcohol for precipitation in the presence of NaCl when $CaCl_2$ is added. This is a much more promising method for the study of antagonistic salt action than the one used by Lenk, but the results of Fenn published in his preliminary notices show that he also studied the effects of salts on protein without first removing the excess of salt so that he missed the ionization effect.

Theoretical Remarks.

The paper gives a new and convenient method for the investigation of the effects of electrolytes on the physical properties of proteins and other so called colloids. This method leads to entirely different results from those obtained by the old method of Hofmeister on the swelling of gelatin, and this difference is due to the fact that in Hofmeister's method the effect of the salt on swelling is observed in the presence of an excess of salt, which, as our method shows, inhibits the additional swelling effect of the salt. This latter is the only characteristic effect of the salt on the swelling. Hence it is not possible to discover by Hofmeister's

[14] Lenk, E., *Biochem. Z.*, 1916, lxxiii, 15, 58.
[15] Fenn, W. O., *Proc. Nat. Acad. Sc.*, 1916, ii, 534, 539.

Digitized by Google

method the true characters of the effect of neutral salts (or any true effect of salts) upon the swelling of gelatin.

The most important result obtained with our method is the proof that the influence of salts upon the swelling of gelatin is of a stoichiometrical character; *i.e.*, we can utilize the limiting concentration of different neutral salts for the additional swelling of sodium gelatinate to ascertain the molecular concentration of the salt. Experiments on the action of salt upon gelatin treated previously with acid or alkali harmonize with the results given in this paper. They will be discussed in a following paper. The fact that dried pig's bladder behaves similarly to the powdered gelatin indicates the more general character of our results.

In the explanation of these phenomena the writer has adopted the idea that it is the degree of ionization of gelatin salts (of the type sodium gelatinate) which determines the additional swelling observed when the gelatin is first treated with a high concentration of an alkali metal salt (M/8 or M/4) and the salt has been washed away. The reasons for this assumption are given in the paper and need not be repeated here; but we may add that the new method and the new stoichiometric facts do not depend upon this hypothesis. If we adopt the ionization hypothesis, which seems supported by the facts known at present and contradicted by none, it follows, from our observations that we have at present two cases in which antagonistic salt action is clearly due to the fact that one electrolyte, *e.g.* NaCl or acid, causes the formation of ionized protein, while the other electrolyte, *e.g.* $CaCl_2$ or neutral salts in general, causes the transformation of ionized into non-ionized protein, or inhibits in some other way the swelling effect of ionization of the protein molecule. The observations on the influence of neutral salts on the diffusion of potassium salts into the egg of *Fundulus* may form a third case.

SUMMARY.

1. A new method has been described which allows us to study the effect of neutral salts on gelatin. The essential part of this method consists in using the protein in powdered form, applying the salt in not too low a concentration (M/8 or M/4), and then washing away the salt solution.

Digitized by Google

2. This method has led to the result that the influence of neutral salts on the swelling of gelatin is of a stoichiometric order. Powdered gelatin, when perfused by M/8 or M/4 solution of NaCl, shows an additional swelling when afterward perfused by a weaker solution of a neutral salt with univalent metal. This *additional* swelling is only possible as long as the weaker solution remains below a certain concentration. A comparison of this critical concentration for neutral salts of univalent cations with univalent and with bivalent anions shows that the limiting molecular concentration for the *additional* swelling is twice as great if the anion is univalent as when it is bivalent; regardless of the nature of the anion and cation. The facts can be best explained on the assumption that the inhibiting effect of the salt upon the additional swelling is due to the diminution of the degree of electrolytic dissociation of a metal-protein compound. If we make this assumption, which is supported by the facts known at present, our observations lead to the following conclusions.

3. Neutral salts with a univalent cation (in concentrations of M/8 or M/4) form highly ionizable salts with gelatin.

4. It seems that these gelatin salts ionize under formation of a positively charged metal ion (that of the salt used) and a negative gelatin ion which may or may not contain the anion of the salt in non-dissociated bondage.

5. The formation of these gelatin ions causes the considerable additional swelling when the gelatin is first treated with the salt and then, after the salt is washed away, is exposed to distilled water.

6. The metals of the alkaline earth group form salts with proteins of the type calcium gelatinate, which are not capable of swelling and perhaps little or not at all of ionization. The transformation of protein salts with univalent cation (type sodium gelatinate) capable of swelling into protein salts with bivalent cation (type calcium gelatinate) not capable of swelling is the cause of the antagonistic action of the metals of the calcium group.

7. These results contradict the conclusions drawn from Hofmeister's experiments on the swelling of gelatin and it is pointed out that he was misled by a method not suited for the purpose.

Digitized by Google

Digitized by Google

A STUDY OF THE NON-PROTEIN NITROGEN OF WHEAT FLOUR.*

By M. J. BLISH.

(*From the Chemistry Department of the Montana Agricultural Experiment Station, Bozeman.*)

(Received for publication, December 26, 1917.)

Preliminary to a projected investigation concerning the more important biochemical changes which occur when wheat is frosted, as well as the relation of these changes to bread-making value, and particularly in order to study the effect of premature freezing on the nitrogen compounds of the wheat kernel, it has been found desirable to develop a more satisfactory method for the separation of protein from non-protein nitrogen compounds in wheat flour than an examination of the literature has revealed. A number of methods have been developed for the separation of protein from non-protein nitrogen in almost all kinds of biological products, and several of these appear to be satisfactory for their purposes. With cereals, however, none of these methods seems to answer the purpose since cereals contain alcohol-soluble proteins, which are not encountered in any other plant or animal tissues.

Reagents ordinarily used for precipitating proteins, such as alcohol, acetic acid, trichloroacetic acid, salts of heavy metals, colloidal iron, aluminum hydroxide cream, phosphotungstic acid, and tannic acid, are for various reasons unsatisfactory for removing gliadin from water extracts of flour.

Ritthausen,[1] 40 years ago, advocated the quantitative removal of the proteins from milk, by alternately adding to the protein in solution dilute copper sulfate and potassium hydroxide until the proportions were such that the copper precipitate would no longer redissolve. The insoluble copper-protein compounds were then

* Published with the approval of the Director of the Montana Agricultural Experiment Station.

[1] Ritthausen, H., *J. prakt. Chem.*, 1872, v, 215.

Digitized by Google

removed by filtration. This principle in modified forms has since been made use of by different investigators, although the accuracy of the separation of protein from non-protein nitrogen by this method has always been questioned.

Osborne and Leavenworth[2] have recently reported a study of copper-protein compounds, using edestin and gliadin in their experiments, and have found that if the correct amount of copper sulfate is added to a solution of gliadin in dilute sodium hydroxide solution, the gliadin and copper are both practically completely precipitated. As they state, however, there are several points to be definitely cleared up if this procedure is to be made a basis for the accurate separation of protein from non-protein nitrogen compounds in extracts from biological material.

When the principle of Osborne's procedure was tried with flour extracts in this laboratory, it was found that more nitrogen was removed by this means than by the use of any other of the reagents previously mentioned. Phosphotungstic acid removed nearly, but not quite, as much and tannic acid slightly less. Moreover, the copper-protein precipitate filtered readily, giving a water-clear solution, which could easily be concentrated under reduced pressure to one-twentieth of its original volume. That there was probably no copper in the filtrate, other than that which was in combination with amino-acids and peptides, was indicated by the fact that no blue color was perceptible, nor were positive tests with potassium ferrocyanide obtained until after considerable concentration. The method seemed so simple of manipulation that it was decided to test the effectiveness of the separation by ascertaining whether or not all protein was removed, as well as to test the copper-protein precipitate for free amino nitrogen as an indication of the presence or absence of less complex nitrogen compounds in the copper-protein precipitate.

In Table I are data which indicate the total nitrogen which is *not* precipitated from a water extract of a standard patent flour (Ceretana) by the copper method, as compared with the total nitrogen *not* removed from the same extract by tannic acid, colloidal iron, and phosphotungstic acid, respectively. The figures represent averages of several determinations by each method.

[2] Osborne, T. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1916-17, xxviii, 109.

Digitized by Google

TABLE I.

Method of treatment using 50 cc. portions of extract.	Total N not precipitated.
	gm.
1. 1 cc. Merck's 5 per cent colloidal iron ppt. at room temperature, followed by 1 cc. of concentrated $MgSO_4$ solution	0.00266
2. Same as No. 1 with precipitation at boiling temperature	0.0033
3. Precipitation with 10 cc. of 10 per cent phosphotungstic acid after making strongly acid with HCl	0.0010
4. Precipitation with tannic acid	0.0012
5. Copper method	0.0008

The details of the copper method as used on flour extracts in this laboratory are presented in the following experiments.

Experiment I.

Ceretana, a standard patent flour milled by the Bozeman Milling Company of Bozeman, Montana, was used in the preliminary work, which consisted of determining the proper proportions of flour to water for the extraction, the length of time of extraction, and a comparison of the copper method with the other methods mentioned earlier in this paper with respect to the "total non-protein nitrogen" determined by other methods. Distilled CO_2-free water, saturated with toluene, was used for all extractions. After trying various proportions of flour to water, 20 parts of water to 1 of flour was decided upon as a convenient proportion to use for extractions. Extractions were carried on for varying lengths of time, using portions of 20 gm. of flour to 400 cc. of water in 500 cc. Erlenmeyer flasks. The flasks were shaken vigorously every 15 minutes, for periods of 2, 3, 4, 5, 6, and 12 hours, respectively. At the end of each period the extract was filtered through paper, the proteins were precipitated by treating 50 cc. of extract with 15 cc. of 0.1 N NaOH, followed by 16 cc. of 0.1 N $CuSO_4$, and total nitrogen was determined in the filtrate from the copper precipitation. The amount was found to be the same for the 2 hour extraction as for the 12 hour period, and therefore a minimum extraction period of 2 hours was adopted. Perhaps the most satisfactory method for the

Digitized by Google

determination of "*total* non-protein nitrogen" in flour extracts by the above method is to pipette 100 cc. of flour extract into a 200 cc. sugar flask, add 25 cc. of 0.1 N NaOH followed by 27 cc. of 0.1 N $CuSO_4$, shake vigorously several times until a water-clear supernatant liquid remains after the precipitate settles, make to the mark, filter, and determine nitrogen by the Kjeldahl method in 100 or 150 cc. of the filtrate.

The effectiveness of the method was then examined by studying the nature of the nitrogen compounds left in the filtrate from the copper-protein precipitation. In this work, two flours, A and B, were used. Flour A was the Ceretana patent flour mentioned previously, while B was a flour milled from some slightly frosted Karkov wheat grown in Montana. 1 liter portions of filtered flour extract were placed in 2 liter flasks, and 400 cc. portions of 0.1 N NaOH were added, followed by 400 cc. of 0.1 N $CuSO_4$. Then small portions (of about 10 cc. each) of 0.1 N $CuSO_4$ were added, the whole being well shaken and allowed to settle after each addition, until an absolutely clear, colorless supernatant liquid remained after the precipitate settled. *It appears to be absolutely necessary that the 0.1 N alkali be kept in paraffin-lined containers, or else be made up fresh for each occasion,* as it was found that alkali containing dissolved glass would neither cause a sharp separation nor give a clear, colorless filtrate. Finally, distilled water was added up to the 2 liter mark, and the filtration performed on a large paper filter. The filtration proceeds rapidly and easily when the precipitation is made in the manner described. The filtrate was then slightly acidified with acetic acid and concentrated under diminished pressure to one-twentieth of its volume. The concentrated solutions were slightly viscous on account of dissolved carbohydrate material, and gave a slight test for copper with ferrocyanide, which they did not give before concentration. They also showed the slightest trace of color due to copper salts. These concentrated solutions are referred to in the remainder of this paper as Solution X. These solutions gave no perceptible biuret reaction of any kind, but did give a very faint Millon's reaction, and also a slight Adamkiewicz' reaction. The latter two reactions are indicative of the presence of the amino-acids, tyrosine and tryptophane, but do not necessarily show that they are in protein combination. Solution X

Digitized by Google

gave a slight color with Nessler's reagent, indicating but a trace of free ammonia. Amino nitrogen was determined in Van Slyke's micro-apparatus using 5 cc. portions of Solution X. Amide nitrogen was determined by diluting 50 cc. of Solution X to 100 cc., boiling for 2 hours with 2.5 cc. of concentrated sulfuric acid, according to the method of König,[3] and distilling with an excess of calcium hydroxide under reduced pressure. In order to find out approximately the nature of the rest of the nitrogen in the extracts, 25 cc. portions of Solution X were hydrolyzed with strong hydrochloric acid for about 12 hours, after which ammonia and amino nitrogen were again determined, and the increase in these constituents over the amounts as determined before hydrolysis was considered as indicative of the nature and complexity of any nitrogen compounds still present in peptide or protein form. In Table II are presented results of analyses of flours A and B.

TABLE II.

Constituent determined.	Flour A (sound patent).	Flour B (slightly frosted).	Flour A.	Flour B.
	per cent	*per cent*	*gm.*	*gm.*
Total N of flour	1.89	1.79		
Per cent of total N of flour extracted by distilled CO_2-free water in 2½ hours	16.06	15.40		
Per cent of total flour N not precipitated by copper	1.62	2.12		
Free ammonia in Solution X	Trace.	Trace.		
Amide N in total "non-protein" N	20.00	26.12		
Gm. of amide N in 100 gm. of flour			0.006	0.0095
α-Amino N in total "non-protein" N	6.62	7.79		
Gm. of amino N in 100 gm. of flour			0.002	0.003
Ammonia N after hydrolysis	10.91	10.32		
α-Amino N increase due to hydrolysis	19.34	20.22		
Humin N after hydrolysis	8.16	7.37		
Residual nitrogen by difference	34.97	28.18		

It will be observed from Table II that the actual quantity of free amino nitrogen in a normal flour is exceedingly small, being about 2 mg. for every 100 gm. of flour. There is about three

[3] König, J., Chemie der menschlichen Nahrungs- und Genussmittel, Berlin, 4th edition, 1910, iii, 274.

Digitized by Google

times as much nitrogen in the acid amide form. The figures for amino nitrogen vary widely ffom those obtained by Swanson and Tague[4] who determined amino nitrogen in a patent flour using Sörensen's formol titration method and report about nine times as much as is found in these experiments. They used flour extracts without attempting to remove the proteins. Van Slyke micro determinations were made directly on some flour A extract in this laboratory in order to obtain a more satisfactory comparison with their method. The presence of the proteins made it impossible to take an adequate concentrated sample so that determinations had to be made using 5 cc. of the extract, corresponding to 0.25 gm. of flour. This, according to the method used by Swanson and Tague, should give about 0.1 cc. of nitrogen gas which may be easily measured in the micro-apparatus. In no case, however, could more than 0.02 cc. be obtained, while the average of four determinations was about 0.01 cc., which is too small an amount to be considered for the purposes of a satisfactory determination, although quite in accordance with the findings using the Van Slyke method on the concentrated extract from which the proteins had previously been precipitated with copper; *i.e.*, Solution X.

Table II shows an increase in ammonia and amino nitrogen in Solution X after prolonged hydrolysis with strong acid. The increase, however, is not great enough to indicate the presence of any considerable amount of protein. It suggests, however, that there is some nitrogen in the form of peptides which escapes precipitation by the copper method, although it does not preclude the possibility that traces of protein may still be present also. Hart and Bentley,[5] studying the non-protein nitrogen of some feeding-stuffs, found that after treatment with Stutzer's reagent some nitrogen of the nature of peptide linkings remained in solution. The same seems to be true when Osborne's procedure is followed, although it is probable that the precipitation of the true proteins is practically complete if the proper manipulation is followed.

[4] Swanson, C. O., and Tague, E. L., *J. Am. Chem. Soc.*, 1916, xxxviii, 1098.

[5] Hart, E. B., and Bentley, W. H., *J. Biol. Chem.*, 1915, xxii. 477.

Digitized by Google

Experiment II.

In order to obtain a more satisfactory idea of the nature of the peptide linkings left in solution after the copper treatment, a portion of Solution X from flour A was acidified and treated with phosphotungstic acid. A considerable precipitate formed which contained 40.91 per cent of the total nitrogen in the solution notwithstanding the fact that when the *original water extract* of flours is treated with phosphotungstic acid no more (and often less) nitrogen is precipitated than by the copper method. However, when Solution X was subjected to a further treatment with copper, as in the first removal of the proteins, no more nitrogen was precipitated. Furthermore, when a portion of Solution X was mixed with about nine times its volume of 95 per cent alcohol, 30 per cent of the total nitrogen in the solution was precipitated, along with the dextrins and other carbohydrates insoluble in alcohol. These facts strongly suggest the presence of peptide linkings of a less complex nature than true protein. The phosphotungstic acid precipitate was filtered off, washed with a solution of phosphotungstic and hydrochloric acids, and hydrolyzed for about 12 hours with strong hydrochloric acid, after which ammonia and amino nitrogen were determined. Amino nitrogen was also determined in the filtrate from the phosphotungstic acid precipitation. Although the determinations were made on small amounts of material, they were confirmed by several closely agreeing duplicates and blanks on each analysis, the result being reported in Table III.

TABLE III.

Constituent determined in terms of total N in Solution X.	Flour A.
	per cent
N precipitated by alcohol	30.00
N precipitated by further treatment with $CuSO_4$ and NaOH.	None.
N precipitated with phosphotungstic acid	40.91
Total amino N in Solution X	6.62
Total amino N in filtrate from phosphotungstic acid precipitation	4.81
Amino N from exposed amino groups of peptides (by difference)	1.81
Ammonia N after hydrolysis of phosphotungstic acid precipitate	5.71
Amino N after hydrolysis	14.30

Digitized by Google

As nearly as may be ascertained from determinations with such small amounts of material as are necessitated in the preceding experiments, the figures reported in Table III show that there cannot have been much, if any, protein left in solution after the first precipitation by the copper method. The amino nitrogen determinations on Solution X before and after precipitation with phosphotungstic acid show that there is 1.81 per cent less amino nitrogen after the precipitation than before. This must be due to the exposed amino groups of the nitrogenous matter precipitated by the phosphotungstic acid, and although the difference involved the measurement of only 0.03 cc. of nitrogen gas in the micro determination, it was confirmed by repeated determinations which agreed practically as closely as the burette could be read. After hydrolysis there was about eight times as much amino nitrogen, which clearly indicates that the precipitate consisted chiefly of peptides of a less complex nature than protein. The amino nitrogen in the filtrate from the treatment of Solution X with phosphotungstic acid which amounted to 4.81 per cent probably originated from the *free* amino-acids in the extract. There is a much larger discrepancy between the percentage of total nitrogen precipitated by phosphotungstic acid and the sum of the percentages of ammonia and amino nitrogen after hydrolysis than may reasonably be accounted for by the non-amino nitrogen of the peptide compounds. This seems to indicate that there are other basic nitrogen compounds in flour extracts, whose nature is as yet largely unknown. A murexide test for purines resulted negatively.

An attempt was made to ascertain whether or not the copper method removes along with the proteins any appreciable amounts of amino-acids or peptide-like compounds of a nature less complex than that of the proteins by redissolving some of the copper-protein precipitate in glacial acetic acid and testing the solution for free amino groups since the presence of considerable copper does not interfere with the reaction between the amino groups of amino-acids and nitrous acid. Accordingly a solution of the copper precipitate containing 4 mg. of nitrogen was introduced into the Van Slyke micro-apparatus, but there was no evidence to show that any such compounds were present in the precipitate. Therefore, it is not believed that any serious error

Digitized by Google

from this source is introduced in the case of flour extracts, notwithstanding the fact that under certain conditions copper is known to be capable of forming insoluble compounds with a few amino-acids, though not with the majority of them.

When water extracts of flour were allowed to stand for several weeks at room temperature in the presence of toluene, and analyzed at intervals for non-protein nitrogen by the copper method, a gradual increase in non-protein nitrogen by autolysis occurred although there was no sign of putrefaction. The amount of total non-protein nitrogen doubled during the period from May 25 to July 6, 1917. This indicates that the method is applicable to proteolysis studies in flour.[6]

SUMMARY.

1. Practically a complete separation of protein from non-protein nitrogenous substances in water extracts of wheat flour may be accomplished by treating the extract with 0.1 N NaOH followed by 0.1 N $CuSO_4$ until there is but slightly more $CuSO_4$ than an exactly equivalent amount of NaOH. The method is simple of manipulation and leaves no troublesome excess of the reagents employed for the precipitation. The method permits of rapid filtration through ordinary filter paper, giving a water-clear solution which may be readily concentrated to one-twentieth its original volume, for determinations of amino nitrogen by Van Slyke's micro method, and for amide nitrogen determinations. Some peptide nitrogen is not precipitated by the copper method, but the removal of the true proteins is practically complete.

2. Normal patent flour contains but about 2 mg. of amino-acid nitrogen for every 100 gm. of flour, and about three times as much nitrogen in free acid amide form.

3. There is probably a considerable amount of *non-protein nitrogen* not precipitated by the copper method which is neither amino-acid nitrogen nor is it in the form of peptide complexes; its nature is not known.

4. The method is applicable to studies of proteolysis or other studies involving the estimation of protein cleavage products in wheat flour.

5. It is not unlikely that the method will be found applicable to biological extracts from other sources than wheat and flour.

[6] Acknowledgment is made to Miss Erma Lessel, who performed a large part of the preliminary analytical work.

Digitized by Google

Digitized by Google

INDEX TO VOLUME XXXIII.

ACID, amino-, nitrogen, estimation in blood, 325
—— and alkaline gelatin, antagonistic effect of salts on the precipitation of, by alcohol, 439
—— and salt, effects of, on the precipitation of gelatin by alcohol, 279
—— excretion, and mathematical formulas, 267
——, fatty, in human blood in normal and pathological conditions, 401
——, phosphoric, penetration of cells by, 463
—— production, studies, 103
—— uridinephosphoric, 229
——, yeast nucleic, structure of, 229, 425
Acidosis in experimental hyperthyroidism and its relation to epinephrine in the blood and the decrease of liver glycogen, 215
——, mineral loss during, 103
——, studies of, 271
VON ADELUNG, EDWARD. See SCHMIDT, VON ADELUNG, and WATSON, 501
Adenine and guanine in cow's milk, 145
Adrenals of thyroid-fed rats, epinephrine content of, 207
Alcohol, antagonistic effect of salts on the precipitation of acid and alkaline gelatin by, 439
——, precipitation of gelatin by, effects of acids and salts on, 279
——, protein soluble in, 243
Alkali and carbon dioxide, high levels of, 333
————————, low levels of, induced by ether, prevention and reversal of, 345
Alkaline gelatin and acid, antagonistic effect of salts on the precipitation of, by alcohol, 439
—— reserve of blood plasma and other frothing fluids, new hydrogen electrode for electrometric titration of, 19
ALMY, LLOYD H. See CLARK and ALMY, 483
Amino nitrogen method, Van Slyke, foam inhibitor in, 373
——-acid nitrogen in the blood, estimation of, 325
Ammonia and urea in placenta tissue, direct determination of, 381
—— hydrolysis of yeast nucleic acid, 425
Analyzer, Haldane gas, 31
Anemia, experimental, studies of the blood fat and lipoids of the dog before and after the production of, 377
Ascaris suum, catalase content, with a suggestion as to its rôle in protecting parasites against the digestive enzymes of their hosts, 395
ATKINSON, H. V. Animal calorimetry. The influence of mechanical work upon protein metabolism during the height of meat digestion in the dog. Fourteenth paper, 379

Digitized by Google

Austin, J. Harold, and Jonas, Leon. Effects of diet on the plasma chlorides and chloride excretion in the dog, 91

Bacterial cells, method for nitrogen content of, 525
Barnett, George D. Mathematical formulas and acid excretion, 267
Bases, guanidine, studies in the metabolic changes induced by administration of, 253
Baumann, L. See Sawyer, Baumann, and Stevens, 103
Biological analysis of pellagra-producing diets, 303, 411
—— significance of effects of electrolytes on gelatin, 279, 439
Blish, M. J. A study of the nonprotein nitrogen of wheat flour, 551
Blood, carbon dioxide capacity of, respiratory regulation, 333, 345, 355, 365
——, epinephrine in, and the decrease of liver glycogen, and relation to acidosis in experimental hyperthyroidism, 215
——, estimation of amino-acid nitrogen in, 325
—— fat and lipoids of the dog before and after the production of experimental anemia, studies of, 377
—— gas analysis, 39
——, glucose and cholesterol content, comparison of, 387
——, human, in normal and pathological conditions, fatty acids in, 401
——, normal human, amount and distribution of creatinine and creatine in, 169
——, occult, method for detection in stools, 1
Blood, oxygen and hemoglobin of, gasometric determination of, 127
—— plasma and other frothing fluids, new hydrogen electrode for electrometric titration of the alkaline reserve of, 19
—— sugar content, influence of injected guanidine hydrochloride upon, 253
——, venous, oxygen in, studies of technique and results on normal individuals, 133
Bradley, Harold C., and Nichols, M. Starr. Nitrogen content of bacterial cells. I. Method, 525
Breathing, excessive, reduced carbon dioxide capacity, shock, and trauma, sequence of, 365
Bromate, potassium, effect upon enzyme action, 453

Calorimetry, animal, 379
——, indirect, and respiratory exchange, 47
Campbell, Walter R. See Hunter and Campbell, 169
Carbohydrate metabolism, influence of thyroid feeding upon, 193, 207, 215
Carbohydrates, specific, and grains, other than oats, influence of, on the development of scurvy, 471
Carbon dioxide and alkali, high levels of, 333
—— —— —— ——, low levels of, induced by ether, prevention and reversal of, 345
—— —— capacity of the blood, respiratory regulation of, 333, 345, 355, 365
—— —— ——, reduced, trauma, excessive breathing, and shock, sequence of, 365

Digitized by Google

Catalase content of *Ascaris suum*, with a suggestion as to its rôle in protecting parasites against the digestive enzymes of their hosts, 395
Cell penetration by phosphoric acid, 463
Cells, bacterial, method for nitrogen content of, 525
Cephalin, hydrolecithin and its bearing on the constitution of, 111
Chemical analysis of twenty common food fishes, with especial reference to seasonal variation in composition, 483
Chlorides, plasma, and chloride excretion in the dog, effects of diet on, 91
Cholesterol and glucose content of the blood, comparison of, 387
CLARK, ERNEST D., and ALMY, LLOYD H. A chemical study of food fishes. The analysis of twenty common food fishes with especial reference to a seasonal variation in composition, 483
Colorimetric determination of hemoglobin, 119
Copper-phosphate mixtures as sugar reagents, 513
Cottonseed oil, peas, and wheat flour, nature of the dietary deficiencies of a diet of, 411
Creatine and creatinine in normal human blood, amount and distribution of, 169
Creatinine and creatine in normal human blood, amount and distribution of, 169
CRETCHER, L. H., JR. See LEVENE and CRETCHER, 505
CROZIER, W. J. Cell penetration by acids. IV. Note on the penetration of phosphoric acid, 463
CSONKA, FRANK A. The fatty acids in human blood in normal and pathological conditions, 401
CURTMAN, LOUIS J. See LYLE and CURTMAN, 1

DANIELS, AMY L., and LOUGHLIN, ROSEMARY. Feeding experiments with peanuts, 295
DENIS, W. See FOLIN and DENIS, 521
Determination, colorimetric, of hemoglobin, 119
——, direct, of urea and ammonia in placenta tissue, notes on, 381
——, gasometric, of oxygen and hemoglobin of blood, 127
—— of amino-acid nitrogen in blood, 325
—— —— lactose in milk, 521
——, sodium and potassium, 235
Diet, effects of, on the plasma chlorides and chloride excretion in the dog, 91
—— of peas, wheat flour, and cottonseed oil, nature of the dietary deficiencies of, 411
——, pellagra-producing, biological analysis of, 303, 411
Dietary deficiencies of a diet derived from peas, wheat flour, and cottonseed oil, 411
—— essential, water-soluble B, study of, in relation to its solubility and stability towards reagents, 55
Digestion of meat in the dog, influence of mechanical work upon protein metabolism during the height of, 379
Digestive enzymes of hosts, catalase content of *Ascaris suum*, with a suggestion as to its rôle in protecting parasites against, 395
—— tract of invertebrates, lichenase in, 161

Digitized by Google

DUBIN, HARRY. Studies of the blood fat and lipoids of the dog before and after the production of experimental anemia, 377

ECKSTEIN, H. C. See MITCHELL and ECKSTEIN, 373

Electrode, hydrogen, for the electrometric titration of the alkaline reserve of blood plasma and other frothing fluids, 19

Electrolytes, effects of, on gelatin and their biological significance, 279, 439

Electrometric titration of alkaline reserve of blood plasma and other frothing fluids, new hydrogen electrode for, 19

Enzyme action, effect of potassium bromate upon, 453

Enzymes, digestive, of hosts, catalase content of *Ascaris suum* with a suggestion as to its rôle in protecting parasites against, 395

Epinephrine content of the adrenals of thyroid-fed rats, 207

—— in the blood and the decrease of liver glycogen, and relation to acidosis in experimental hyperthyroidism, 215

Ether production of low levels of carbon dioxide and alkali, prevention and reversal of, 345

FALK, I. S., and WINSLOW, C.-E. A. The effect of potassium bromate upon enzyme action, 453

Fat, blood, and lipoids of the dog before and after the production of experimental anemia, studies of, 377

Fatty acids in human blood in normal and pathological conditions, 401

Feeding experiments with peanuts, 295

Feeding, thyroid, influence upon carbohydrate metabolism, 193, 207, 215

FENN, W. O. The effects of electrolytes on gelatin and their biological significance. I. The effects of acids and salts on the precipitation of gelatin by alcohol, 279. II. The effects of salts on the precipitation of acid and alkaline gelatin by alcohol. Antagonism, 439

Fishes, food, chemical analysis of twenty common, with especial reference to seasonal variation in composition, 483

Flour, wheat, peas, and cottonseed oil, nature of the dietary deficiencies of a diet of, 411

——, ——, study of the non-protein nitrogen of, 551

Fluids, frothing, and blood plasma, new hydrogen electrode for electrometric titration of the alkaline reserve of, 19

Foam inhibitor in the Van Slyke amino nitrogen method, 373

FOLIN, OTTO, and DENIS, W. The determination of lactose in milk, 521

—— and MCELLROY, W. S. Copper-phosphate mixtures as sugar reagents. A qualitative test and a quantitative titration method for sugar in urine, 513

Food fishes, a chemical analysis of twenty common, with especial reference to seasonal variation in composition, 483

Formulas, mathematical, and acid excretion, 267

GAS analysis, applications of, 31, 39, 47

—— ——, blood, 39

—— analyzer, Haldane, 31

Digitized by Google

Gasometric determination of oxygen and hemoglobin of blood, 127
Gelatin, alkaline and acid, antagonistic effect of salts on the precipitation of, by alcohol, 439
——, effects of electrolytes on, and and their biological significance 279, 439
——, precipitation of, by alcohol, effects of acids and salts on, 279
Glucose and cholesterol content of the blood, comparison of, 387
Glycogen, liver, decrease of, and epinephrine in blood, and relation to acidosis in experimental hyperthyroidism, 215
——, ——, in thyroid-fed animals, storage and mobilization of, 193
Grains, other than oats, and specific carbohydrates, influence on the development of scurvy, 471
Growth in young animals, causes of failure of mixtures of seeds to promote, 303
—— of chickens in confinement, 433
Guanidine bases, studies in the metabolic changes induced by administration of, 253
—— hydrochloride, injected, influence of upon blood sugar content, 253
Guanine and adenine in cow's milk, 145

HAGGARD, H. W. See HENDERSON and HAGGARD, 333, 345, 355, 365
Haldane gas analyzer, 31
HAMMETT, FREDERICK S. Notes on the direct determination of urea and ammonia in placenta tissue, 381
HART, E. B., and STEENBOCK, H. Thyroid hyperplasia and the relation of iodine to the hairless pig malady. I, 313
Hemoglobin and oxygen of blood, gasometric determination of, 127
——, colorimetric determination of, 119
HENDERSON, YANDELL. Applications of gas analysis. IV. The Haldane gas analyzer, 31. VI. The respiratory exchange and indirect calorimetry, 47
—— and HAGGARD, H. W. Respiratory regulation of the CO_2 capacity of the blood. I. High levels of CO_2 and alkali, 333. II. Low levels of CO_2 and alkali induced by ether. Their prevention and reversal, 345. III. The effects of excessive pulmonary ventilation, 355. IV. The sequence of trauma, excessive breathing, reduced CO_2 capacity, and shock, 365
—— and SMITH, ARTHUR H. Applications of gas analysis. V. Blood gas analysis, 39
HILL, R. L. The analysis of milk secreted by a suckling doe kid, 391
Histidine, method for the preparation of, 429
HOGAN, ALBERT G. The nutritive properties of kafirin, 151
HUNTER, ANDREW, and CAMPBELL, WALTER R. The amount and the distribution of creatinine and creatine in normal human blood, 169
Hydrocarbons, tertiary, synthesis and oxidation of, 505
Hydrochloride, guanidine, injected, influence of, upon blood sugar content, 253
Hydrogen electrode for the electrometric titration of the alkaline reserve of blood plasma and other frothing fluids, 19

Digitized by Google

Hydrolecithin and its bearing on the constitution of cephalin, 111
Hydrolysis, ammonia, of yeast nucleic acid, 425
Hyperplasia, thyroid, and relation of iodine to the hairless pig malady, 313
Hyperthyroidism, experimental, acidosis in, and its relation to epinephrine in the blood and the decrease of liver glycogen, 215

INHIBITOR, foam, in the Van Slyke amino nitrogen method, 373
Invertebrates, digestive tract of, occurrence of lichenase in, 161
Iodine, relation to thyroid hyperplasia and the hairless pig malady, 313
Ionization of proteins and antagonistic salt action, 531

JEWELL, MINNA E., and LEWIS, HOWARD B. The occurrence of lichenase in the digestive tract of invertebrates, 161
JONAS, LEON. See AUSTIN and JONAS, 91
JONES, HORRY M. A detailed method for the preparation of histidine, 429

KAFIRIN, nutritive properties of, 151
KURIYAMA, SHIGENOBU. The influence of thyroid feeding upon carbohydrate metabolism. I. The storage and mobilization of the liver glycogen in thyroid-fed animals, 193. II. The epinephrine content of the adrenals in thyroid-fed rats, 207. III. The acidosis in experimental hyperthyroidism and its relation to epinephrine in the blood and the decrease of liver glycogen, 215

LACTOSE in milk, determination of, 521
Lecithin (hydrolecithin), its bearing on the constitution of cephalin, 111
LEVENE, P. A. The structure of yeast nucleic acid. II. Uridinephosphoric acid, 229. III. Ammonia hydrolysis, 452
—— and CRETCHER, L. H., JR. Synthesis and oxidation of tertiary hyrocarbons, 505
—— and WEST, C. J. Lecithin, I. "Hydrolecithin" and its bearing on the constitution of cephalin, 111
LEWIS, HOWARD B. See JEWELL and LEWIS, 161
Lichenase, in the digestive tract of invertebrates, 161
Lipoids and blood fat of the dog before and after the production of experimental anemia, studies of, 377
Liver glycogen, decrease of, and epinephrine in blood, relation to acidosis in experimental hyperthyroidism, 215
—— —— in thyroid-fed animals, storage and mobilization of, 193
LOEB, JACQUES. Ionization of proteins and antagonistic salt action, 531
LOUGHLIN, ROSEMARY. See DANIELS and LOUGHLIN, 295
LUNDSGAARD, CHRISTEN. Studies of oxygen in the venous blood. I. Technique and results on normal individuals, 133
LYLE, WILLIAM G., and CURTMAN, LOUIS J. A new method for the detection of occult blood in stools, 1

Digitized by Google

MAGATH, THOMAS BYRD. The catalase content of *Ascaris suum*, with a suggestion as to its rôle in protecting parasites against the digestive enzymes of their hosts, 395
McCLENDON, J. F. A new hydrogen electrode for the electrometric titration of the alkaline reserve of blood plasma and other frothing fluids, 19
McCOLLUM, E. V., and SIMMONDS, N. A biological analysis of pellagra-producing diets. IV. The causes of failure of mixtures of seeds to promote growth in young animals, 303
—— and ——. A study of the dietary essential, water-soluble B, in relation to its solubility and stability towards reagents, 55
——, ——, and PARSONS, H. T. A biological analysis of pellagra-producing diets. V. The nature of the dietary deficiencies of a diet derived from peas, wheat flour, and cottonseed oil, 411
McCRUDDEN, F. H., and SARGENT, C. S. The determination of sodium and potassium, 235
—— and ——. Comparison of the glucose and cholesterol content of the blood, 387
McELLROY, W. S. See FOLIN and McELLROY, 513
MENDEL, LAFAYETTE B. See OSBORNE and MENDEL, 433
Metabolic changes, studies in, induced by administration of guanidine bases, 253
Metabolism, carbohydrate, influence of thyroid feeding upon, 193, 207, 215
——, protein, influence of mechanical work upon, during the height of meat digestion in the dog, 379
Method for detection of occult blood in stools, 1
—— —— preparation of histidine, 429
—— —— —— —— taurin in large quantities, 499
——, quantitative titration, and qualitative test for sugar in urine, 513
——, Van Slyke amino nitrogen, foam inhibitor in, 373
Milk, cow's, adenine and guanine in, 145
——, ——, proteins of, 7
——, determination of lactose in, 521
——, new constituents of, 243
—— secreted by a suckling doe kid, analysis of, 391
Mineral loss during acidosis, 103
MITCHELL, H. H., and ECKSTEIN, H. C. A foam inhibitor in the Van Slyke amino nitrogen method, 373

NICHOLS, M. STARR. See BRADLEY and NICHOLS, 525
Nitrogen, amino, Van Slyke method, foam inhibitor in, 373
——, amino-acid, in blood, estimation of, 325
—— content of bacterial cells, method for, 525
——, non-protein, of wheat flour, study of, 551
Nucleic acid, yeast, structure of, 229, 425
Nutritive properties of kafirin, 151

OKADA, SEIZABURO. On the estimation of amino-acid nitrogen in the blood, 325
OSBORNE, THOMAS B., and MENDEL, LAFAYETTE B. The growth of chickens in confinement, 433

Digitized by Google

Osborne, Thomas B., and Wakeman, Alfred J. The proteins of cow's milk, 7
—— and ——. Some new constituents of milk. Third paper. A new protein, soluble in alcohol, 243
Oxidation and synthesis of tertiary hydrocarbons, 505
Oxygen and hemoglobin of blood, gasometric determination of, 127
—— in the venous blood, studies of technique and results on normal individuals, 133

Palmer, Walter W. The colorimetric determination of hemoglobin, 119
Parasites, protection against digestive enzymes of their hosts, rôle of catalase content of *Ascaris suum* in, 395
Parsons, H. T. See McCollum, Simmonds, and Parsons, 411
Peanuts, feeding experiments with, 295
Peas, wheat flour, and cottonseed oil, nature of the deficiencies of a diet of, 411
Pellagra-producing diets, biological analysis of, 303, 411
Phosphate, copper-, mixtures as sugar reagents, 513
Phosphoric acid, penetration of cells by, 463
Pitz, W. Studies of experimental scurvy. II. The influence of grains, other than oats, and specific carbohydrates on the development of scurvy, 471
Placenta tissue, direct determination of urea and ammonia in, 381
Plasma, blood, and other frothing fluids, new hydrogen electrode for electrometric titration of the alkaline reserve of, 19
Plasma, chlorides and chloride excretion in the dog, effects of diet on, 91
Potassium and sodium, determination of, 235
—— bromate, effect upon enzyme action, 453
Protein metabolism, influence of mechanical work upon, during the height of meat digestion in the dog, 379
——, non-, nitrogen of wheat flour, study of, 551
——, soluble in alcohol, 243
Proteins, ionization of, and antagonistic salt action, 531
—— of cow's milk, 7
Pulmonary ventilation, the effects of excessive, 355

Reserve, alkaline, of blood plasma and other frothing fluids, new hydrogen electrode for electrometric titration of, 19
Respiratory exchange and indirect calorimetry, 47
—— regulation of the CO_2 capacity of the blood, 333, 345, 355, 365

Salt and acid, effects of, on the precipitation of gelatin by alcohol, 279
——, antagonistic action, and ionization of proteins, 531
——, —— effect of, on the precipitation of acid and alkaline gelatin by alcohol, 439
Sargent, C. S. See McCrudden and Sargent, 235, 387
Sawyer, Margaret, Baumann, L., and Stevens, F. Studies of acid production. II. The mineral loss during acidosis, 103
Schmidt, Carl L. A., von Adelung, Edward, and Watson, Thomas. On the elimination of taurin administered to man, 501

Digitized by Google

SCHMIDT, CARL L. A., and WATSON, THOMAS. A method for the preparation of taurin in large quantities, 499
Scurvy, experimental, studies of, 471
——, influence of grains, other than oats, and specific carbohydrates on the development of, 471
Seed mixtures, causes of failure of, to promote growth in young animals, 303
SHERWIN, CARL P. See VOEGTLIN and SHERWIN, 145
Shock, trauma, excessive breathing, and reduced carbon dioxide capacity, sequence of, 365
SIMMONDS, N. See MCCOLLUM and SIMMONDS, 55, 303
——. See MCCOLLUM, SIMMONDS, and PARSONS, 411
SMITH, ARTHUR H. See HENDERSON and SMITH, 39
Sodium and potassium, determination of, 235
STEENBOCK, H. See HART and STEENBOCK, 313
STEVENS, F. See SAWYER, BAUMANN, and STEVENS, 103
Stools, occult blood in, method for detection of, 1
Sugar content, blood, influence of injected guanidine hydrochloride upon, 253
—— in urine, qualitative test and quantitative titration method for, 513
—— reagents, copper-phosphate mixtures as, 513
Suum, Ascaris, catalase content, with a suggestion as to its rôle in protecting parasites against digestive enzymes of their hosts, 395

TAURIN administered to man, elimination of, 501
Taurin, method for preparation in large quantities, 499
Thyroid-fed animals, storage and mobilization of liver glycogen in, 193
—— rats, epinephrine content of the adrenals of, 207
Thyroid feeding, influence of, upon carbohydrate metabolism, 193, 207, 215
—— hyperplasia and the relation of iodine to the hairless pig malady, 313
Tissue, placenta, direct determination of urea and ammonia in, 381
Titration, electrometric, of alkaline reserve of blood plasma and other frothing fluids, new hydrogen electrode for, 19
—— method, quantitative, and qualitative test for sugar in urine, 513
Trauma, excessive breathing, reduced carbon dioxide capacity, and shock, sequence of, 365

UREA and ammonia in placenta tissue, the direct determination of, 381
Uridinephosphoric acid, 229
Urine, qualitative test and quantitative titration method for sugar in, 513

VAN SLYKE amino nitrogen method, foam inhibitor in, 373
VAN SLYKE, DONALD D. Gasometric determination of the oxygen and hemoglobin of blood, 127
——. Studies of acidosis. X, 271
Venous blood, oxygen, studies of, technique and results on normal individuals, 133
Ventilation, excessive pulmonary, effects of, 355
VOEGTLIN, CARL, and SHERWIN, CARL P. Adenine and guanine in cow's milk, 145

Digitized by Google

WAKEMAN, ALFRED J. See OSBORNE and WAKEMAN, 7, 243
WATANABE, C. K. Studies in the metabolic changes induced by administration of guanidine bases. I. Influence of injected guanidine hydrochloride upon blood sugar content, 253
Water-soluble B, dietary essential, study of its solubility and stability towards reagents, 55
WATSON, THOMAS. See SCHMIDT, VON ADELUNG, and WATSON, 501
WATSON, THOMAS. See SCHMIDT and WATSON, 499
WEST, CLARENCE J. See LEVENE and WEST, 111
Wheat flour, peas, and cottonseed oil, nature of the dietary deficiencies of a diet of, 411
—— ——, study of the non-protein nitrogen of, 551
WINSLOW, C.-E. A. See FALK and WINSLOW, 453

YEAST nucleic acid, structure of, 229, 425

Digitized by Google

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

TWELFTH ANNUAL MEETING.

Minneapolis and Rochester, Minn., December 27–29, 1917.

Digitized by Google

Digitized by Google

THE RÔLE OF SOME INORGANIC ELEMENTS IN NUTRITION.

By LAFAYETTE B. MENDEL and THOMAS B. OSBORNE.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

The relative importance of the individual inorganic salts was long ago determined for plants, but hitherto no such definite knowledge of the requirements of animals has been possible owing to the failure of animals to thrive on a diet of purified foodstuffs. Now that the essential factors of a successful dietary have been ascertained it has become possible to prepare rations in which the individual ions are excluded except for the unavoidable small amounts present in the products used to supply the water-soluble vitamine or found as impurities in the other ingredients of the food.

From experiments with such diets it has been demonstrated that calcium and phosphorus are needed in considerable amounts; for with only a trace of either of these elements in the food, rats failed to grow and sooner or later declined. On diets in which only supposedly insignificant quantities of magnesium, sodium, potassium, or chlorine were present growth at a normal or nearly normal rate was made for about 1 year. Where both sodium and potassium were present in the above small amounts no growth occurred.

THE NUTRITIVE PROPERTIES OF KAFIR.

By ALBERT G. HOGAN.

(From the Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan.)

Our investigations of the nutritive properties of kafir were interrupted, and as some time may elapse before this study can be resumed we deem it advisable to take this opportunity of making a preliminary report. Although we have a high degree of

Digitized by Google

confidence in the correctness of our conclusions, these observations are not to be considered as necessarily final.

When kafir forms the sole diet of white rats, it has about the same nutritional value as corn. Kafir is seriously deficient in calcium, but when this element (Ca lactate) is supplied, the animals grow and attain maturity, though the growth period is considerably prolonged. Growth on kafir and ash mixture is apparently more rapid than on corn and ash mixture.

Females receiving kafir and a salt mixture became pregnant and delivered young, but in no case did these litters survive more than a few days.

The proteins of kafir seemed to have a biological value equal to that of corn in inducing growth.

Kafir is probably deficient in one of the accessory substances. A comparison of the data obtained by feeding kafir and by feeding corn indicates that the fat-soluble A, as described by McCollum, is a limiting factor in kafir.

Our investigations on the nutritive properties of kafirin were brought more nearly to completion and the data has been published in detail.[1] Kafirin, when it forms the sole source of protein in the diet, is apparently inadequate for growth or for the maintenance of young animals (rats). Lysine is the first limiting factor of kafirin, and cystine is the second. The results indicate that lysine is indispensable not only for growth, but also for the maintenance of young animals.

THE RELATION BETWEEN PHOSPHORUS METABOLISM AND THE SECRETION OF MILK FAT.

By EDWARD B. MEIGS and N. R. BLATHERWICK.

(*From the Bureau of Animal Industry, United States Department of Agriculture, Washington.*)

Blood samples have been obtained nearly simultaneously from the jugular and mammary veins of milking cows, and the plasma from such samples has been analyzed for total, lipoid, and inorganic phosphorus, the object being to determine whether the concentrations of these kinds of phosphorus were appreciably

[1] Hogan, A. G., *J. Biol. Chem.*, 1918, xxxiii, 151.

Digitized by Google

changed during the passage of the blood through the active gland.

In the earlier experiments the cow was disturbed by precautions which were afterward found to be unnecessary, and the results of the experiments differed according as the subjects were much or little disturbed before and during the collection of the mammary blood. When they were much disturbed, the mammary plasma had the same concentration of lipoid phosphorus as the jugular, but a higher concentration of inorganic phosphorus, and a correspondingly higher concentration of total phosphorus. When they were little disturbed, the mammary plasma had a lower concentration of lipoid phosphorus and a higher concentration of inorganic phosphorus than the jugular.

These results are taken to mean that the precursor in plasma of both milk fat and milk phosphorus is either lecithin or some related compound. The ratio phosphorus: fat in lecithin is about 1: 18, whereas it is a out 1: 50 in milk. If, therefore, the mammary gland takes from the plasma enough lecithin or enough of some related phosphatide body to supply its milk with fat, it gets with it more phosphorus than can be used for the milk, and the excess must be returned to the blood. These considerations are taken to explain the back-flow of inorganic phosphorus from the mammary gland to the blood, which occurred in all the experiments. The experiments indicate further that the disturbance of milking animals interferes with the taking up of phosphatide by the mammary gland, but not with the back-flow of inorganic phosphate from the gland to the blood.

THE EFFECT OF COTTONSEED FLOUR ON ANIMALS, WITH PARTICULAR REFERENCE TO THE INORGANIC CONSTITUENTS OF WHITE RATS.

By F. C. COOK.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Six separate groups of animals, each group consisting of twenty-four rats, twelve mice, and six guinea pigs, were fed for periods up to 6 months on (1) 25 per cent cottonseed flour bread, (2) 25 per cent cottonseed flour dough, (3) 40 per cent cottonseed

Digitized by Google

flour bread, (4) white flour bread, (5) entire wheat bread, and (6) mixed diet.

The exclusive bread diets did not support life, although no pathological lesions were found to be characteristic of any of the diets. 25 per cent cottonseed flour dough supported life longer than 25 per cent cottonseed flour bread. The animals fed entire wheat bread and white bread lived no longer than the animals fed the cottonseed flour breads.

Less nitrogen was required to maintain rats and mice at constant weight when white bread was fed than when entire wheat bread was fed. The largest amount of nitrogen required to maintain constant weight was in the case of the animals fed 40 per cent cottonseed flour bread. These findings are in line with the results reported in the literature for larger animals.

After 6 months, when the experiment closed, the bodies of twenty-four rats, four from each of the six groups, were separately analyzed for inorganic constituents.

The 40 per cent cottonseed flour bread was highest in total ash, P_2O_5, CaO, and MgO of any of the breads fed. The ash constituents were apparently not properly balanced as the bodies of the rats fed on this bread were low in total ash and especially in P_2O_5-free ash. The ratio of MgO to CaO was 1: 15 for the bodies of the rats fed on 40 per cent cottonseed flour bread and 1: 25 for the controls.

The exclusive feeding of cottonseed flour bread apparently caused changes in the ash constituents of the tissues of the rats analyzed. These changes appeared in the results for the rats fed 25 per cent cottonseed flour bread and dough and were marked in the cases of the rats fed 40 per cent cottonseed flour bread.

BORON. ITS EFFECT ON CROPS AND ITS DISTRIBUTION IN PLANTS AND SOILS IN VARIOUS PARTS OF THE UNITED STATES.

By F. C. COOK and J. B. WILSON.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Borax and colemanite (calcium borate) were found to be effective larvicides for the house-fly.[2] To determine the effect of the

[2] Cook, F. C., Hutchison, R. H., and Scales, F. M., *U. S. Dept. Agric., Bull.* 118, 1914.

Digitized by Google

boron, added to manure for this purpose, on plant growth a study was made extending over 3 years in which crops were grown in different parts of the country on soil fertilized with manure containing definite amounts of added borax and of added colemanite. In order to test the cumulative effect of the boron the same plants were treated with manure and boron for three seasons. Wheat, rye, oats, beets, tomatoes, cowpeas, lettuce, soy beans, string beans, potatoes, spinach, kale, cabbage, turnips, corn, and peach trees were included in the experiments.

Practically the same amount of boron was absorbed by the plants whether it was added to the soil as soluble borax or as insoluble colemanite. Wheat, oats, and rye absorbed little boron, while leguminous and succulent plants absorbed comparatively large amounts. There was a marked difference in the distribution of the boron in the roots, tops, and fruit of the different plants. Leguminous plants were very sensitive to the boron. At Bethesda, Maryland, 0.0044 per cent H_3BO_3 added as borax and 0.0058 per cent added as colemanite to the upper 6 inches of soil caused no injury to lettuce, spinach, and kale, while at Arlington, Virginia (5 miles distant), the same amounts of boron gave a reduced yield of these crops. A similar difference in the effect of a definite amount of boron on other plants when grown on different soils is indicated. There is a decided difference in soils in rendering the added boron non-toxic, although all soils tested gradually rendered the soluble boron insoluble and non-toxic. In many soils there is a tendency for plants to absorb boron in proportion to the amounts added to the soil while in other soils the same amounts of boron were absorbed irrespective of the amounts added. The absorption and the toxic effect of boron on plants varies with the variety of plants, the solubility of the boron compound, the amount of the boron compound added to the soil, the time elapsing after the compound is mixed with the soil before planting, the amount of rainfall, etc., and finally with the character of the soil to which the boron compound is added.

Digitized by Google

THE INFLUENCE OF DIET ON THE URINARY CALCIUM AND MAGNESIUM EXCRETION OF MAN.

By MAURICE H. GIVENS.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

Nelson and Burns[3] found that eight out of twenty-five persons, on random diets, excreted more magnesium than calcium in the urine. If one accepts the generally quoted statement that calcium and magnesium excretion is dependent upon the quality and quantity of nourishment ingested, their findings ought to be corroborated with subjects on diets having a content of magnesium greater than that of calcium. Accordingly nine healthy individuals consumed foods estimated to contain more magnesium than calcium for periods of 6 to 9 days. In only three cases was the urinary magnesium excretion greater than that of calcium and in no one of them was the excess of magnesium over calcium greater than 30 mg. per day. In two of these three individuals the urinary calcium output became greater than the magnesium by the addition to the daily intake of two glasses of milk; in the third case a similar result was obtained with a small dose of calcium lactate. When milk was added to the régime the calcium excretion was increased at once with a slight rise in the magnesium.

ACIDOSIS IN RELATION TO PANCREATIC DIABETES.

By V. W. JENSEN.

(From the Northwestern University Medical School, Chicago.)

Elias[4] states that the introduction of acids into rabbits and dogs leads to hyperglycemia and glycosuria. Watanabe[5] in a recent article had occasion to test this out on rabbits and concludes that the results are not at all uniform, and he found that the amount of sugar in the blood was not perceptibly increased. He did not test the urine, but there was no reason to believe that

[3] Nelson, C. F., and Burns, W. E., *J. Biol. Chem.*, 1916–17, xxviii, 237

[4] Elias, H., *Biochem. Z.*, 1913, xlviii, 120.

[5] Watanabe, C. K., *Am. J. Physiol.*, 1917, xlv, 30.

Digitized by Google

there would have been any glycosuria, because of the low amount of sugar in the blood.

The present investigation was commenced without knowledge of this latter work and with the idea of deciding whether or not acid is a cause of glycosuria or a result of a diabetic condition. A great criticism of Elias' work is that the acid he used was too strong, 0.25 N HCl. It is quite possible that his results will hold good, but they are open to the objection that it might have been an indirect action on the pancreas, or a corrosive action on the duodenum.

In the present work, I used 0.1 N HCl, 600 cc. given in 200 cc. doses three times a day. This acid is close to the strength normally found in the dog's stomach. This was tried on two dogs. One dog was rendered glycosuric by removing seven-eighths of the pancreas, and the other highly sensitized by removing six-sevenths of the pancreas. The sensitized dog in no case gave glycosuria by addition of acid, although he could have been rendered highly glycosuric on carbohydrate diet, but was free from it on a meat diet. The glycosuric dog ran on the average 7 gm. of sugar a day on meat diet. During the acid period this amount was doubled. When an equal amount of sodium carbonate was given, the sugar amount dropped to 0.25 gm. per day.

While these experiments are too few in number to give any definite conclusions, it would seem at the present time that acidosis is a result of a glycosuric condition and that after it has developed it acts as a vicious circle and increases the condition. This statement is supported by the fact that when a non-glycosuric sensitized dog is fed a carbohydrate diet, he eliminates 25 gm. of sugar per day, and the acid in urine is doubled, and ammonia excretion is greatly increased.

THE ACTION OF AMMONIUM COMPOUNDS ON PTYALIN.

By ELBERT W. ROCKWOOD.

(From the Department of Chemistry, University of Iowa, Iowa City.)

Digestion was carried on in a medium neutral to litmus. Both salts of inorganic and organic acids were tested. The ammonium salts of the inorganic acids increased the activity of the ptyalin as did also those of the stronger organic acids. In general the

Digitized by Google

ammonium salts of the strong acids had a greater activating effect than those of the weak acids. To this oxalic acid is an exception, having little or no effect.

LIPEMIA.

By W. R. BLOOR.

(From the Biochemical Laboratory of Harvard Medical School, Boston.)

A study was made of the blood lipoids in a case of severe diabetic lipemia where the blood lipoids reached a very high value (over 13 per cent). Analyses were made of blood samples of which the first was taken 36 hours after the last meal and the others at intervals for about 30 days. In the first 20 days of this period no fat was eaten and the total food intake did not exceed 350 calories per day. In the beginning there was marked "diabetic anemia," the blood corpuscle percentage in the first sample being 29 per cent while the normal for this individual was about 42 per cent. During the first 5 days there was considerable acidosis—low CO_2 tension in the alveolar air, high urinary ammonia, and considerable quantities of acetone bodies in the urine—and during this period there was very little absorption of lipoids from the blood; in fact, as a result of the disappearance of the "anemia" and the accompanying concentration of the plasma the lipoid values became higher than at the beginning. The acidosis disappeared during the next 5 day period and with its disappearance there was a rapid lowering of the blood lipoids—about 50 per cent of the total lipoids in the 5 days. Of the lipoids, the fat decreased most rapidly, the lecithin next, and the cholesterol least. The rate of disappearance of the cholesterol was fairly uniform throughout, not being much affected by the presence or absence of acidosis. Changes in the fat and lecithin content of the blood corpuscles were marked throughout, while the cholesterol content remained quite constant, bearing out the earlier findings that the blood corpuscles take an active part in the metabolism of fat and lecithin but not of cholesterol. During the remainder of the period of examination the blood lipoids continued to decrease slowly. The addition of 50 to 60 gm. of fat to the daily diet and the increase of the diet to over 1,000 calories produced only a slight lessening of the rate of decrease.

Digitized by Google

FURTHER STUDIES IN AUTOLYSIS.

By H. C. BRADLEY.

(*From the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.*)

I. Brain.—The autolysis of nervous tissue is accelerated by acids and inhibited by alkali just as are other gland tissues. While the amount of protein present in nervous tissue is very small, the rapidity of its autolysis is about equal to that of liver or kidney. Because of the exceedingly delicate protein framework of the nerve cell, its destruction through the autolytic mechanism may quickly proceed to an irreparable extent, while other organs with their large protein content may undergo large atrophic changes without irreparable damage.

II. Muscle.—Of the three types of muscle tissue, striated autolyzes most slowly and to the smallest extent. Cardiac and smooth muscle autolyze from 50 to 100 per cent faster. All three are found to be accelerated by acidity and inhibited by alkalinity, but to a much less striking extent than the gland tissues previously studied.

III. Colloids.—The work of Ascoli and Izar on the accelerating action of inorganic hydrosols on autolyzing liver was repeated, but not confirmed. Sols of silver, gold, and platinum, prepared by the Bredig method, were found to be wholly inert, neither accelerating nor inhibiting autolysis. Colloidal $Fe(OH)_3$, As_2S_3, and Sb_2S_3 when dialyzed free from electrolytes were found equally inert when added to autolyzing liver pulp. The disagreement between these results and those of the above mentioned authors is so complete that further experiments are under way.

THE INFLUENCE OF MECHANICAL WORK UPON PROTEIN METABOLISM DURING THE HEIGHT OF MEAT DIGESTION IN THE DOG.

By H. V. ATKINSON.

(*From the Physiological Laboratory, Cornell University Medical College, New York City.*)

A dog was given 600 gm. of meat daily in one portion, and the nitrogen eliminated in the urine during the 4th, 5th, and

Digitized by Google

6th hours was determined. The dog was then caused to run on a treadmill during the 5th hour and it was found that the quantity of nitrogen in the urine was the same as during rest. Therefore, mechanical work has no influence on the rate of absorption per hour or the intensity of protein metabolism per hour in a dog which has been given meat in large quantity.

METABOLIC STUDY OF α-UREIDOISOBUTYLACETIC ACID.

By ALICE ROHDE.

(From the Hooper Foundation, San Francisco.)

The procedure described for the identification of α-ureido-β-phenylpropionic acid in the presence of amino-acids and of urea is found to be applicable to the identification of α-ureido-isobutylacetic acid under similar conditions. This uramino-acid was recovered in considerable quantity from the urine of a cat after slow, continuous injection of a saline solution of the material into the blood stream of the animal.

CHANGES IN THE COMPOSITION OF MUSCLE OF SALMON DURING MIGRATION.

By CARL H. GREENE.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

The muscles of the salmon during the fast of the spawning migration show a great decrease in the amount of fat and protein present, with a corresponding increase in the water content. During the first part of the migration the fat shows the greatest decrease but with the beginning of the disappearance of the fat the protein catabolism becomes marked. On the spawning grounds the fish show great exhaustion of all food reserves in comparison to the initial condition.

The organic extractives as a whole and the extractive nitrogen in particular show a general tendency to increase during the migration fast. There is no apparent relationship in the behavior of the organic and inorganic extractives.

The amino nitrogen in the fat-free tissue increases from 61 to 84 mg. per 100 gm. This increase is most marked calculated on

Digitized by Google

the basis of the relation between the amino nitrogen and the protein from which it must be formed. Proportionately, the increase is nearly 100 per cent. During the migration the amino-acids are liberated at the expense of an ever increasing wastage of the muscle proteins.

The most significant change lies in the relation of the amino-acids to the water in the muscle. At the beginning the amino nitrogen value calculated per 100 cc. of water increases from 80 to 100 mg. Its concentration then remains unaffected by subsequent changes. With the stimulated protein catabolism the muscles, or more particularly the fluid in the muscles, become saturated with amino nitrogen early in the migration period. This saturation continues through the migration regardless of the great loss of muscle protein, until in the end the exhaustion of the organism proceeds to a degree culminating in death.

The determining factor in the behavior of the amino-acids would seem to be the volume of water present in the muscles and not the protein. This previously undescribed water volume effect is a strong regulating factor and must be added to those suggested by Van Slyke as probably controlling the amino-acid content of the muscles.

THE COMPOSITION OF THE OVARIES OF THE SALMON DURING MIGRATION.

By C. W. GREENE.

(From the Department of Physiology, University of Missouri, Columbia.)

Chemical analyses were made of the ovaries of fifteen individual salmon of the species *Oncorhynchus tschawytscha* chosen from five stations of the Columbia River basin during the migration to the spawning grounds. The analytical results, with the exception of the fat, were computed in terms of the fat-free sample, and yield the following comparisons. The neutral fat in the wet sample of ovarian tissue averages 14.15 per cent at the mouth of the river, 17.38 per cent about 210 miles up the river, and drops to 10.38 per cent at the spawning grounds. This fat is a superload in the ovules and cannot be considered a part of the ovarian protoplasm. Leaving the fat out of consideration and calculating on a fat-free or protoplasmic basis, the total

Digitized by Google

proteins average 29.8 to 31.2 on the lower river, 31.3 in the upper river, and 29.4 at the spawning grounds, a remarkably constant composition. The lipoids decrease from an average of 4.8 at the mouth of the river to 3.3 at the upper river, and 2.85 on the spawning grounds, showing that the percentage of lipoids diminishes with development, at least in this fasting fish. The organic extractives slightly increase during the early stages of migration but decrease during late migration and at spawning. The inorganic salts increase with the migration. Summarizing, it is apparent that the reserve fat of the ovaries decreased with the migration as does also the percentage amount of lipoids present. The organic extractives vary in harmony with similar changes in other tissues of the body though less in amount. The protein content of this growing reproductive tissue remains remarkably constant. In view of the great increase in mass of the ovaries during the migration and fast, from 600 to 1,000 per cent increase, we must look to other tissues for the food supply used in the growth of the ovaries. Analyses already presented show that the muscles of the salmon contribute this material. The muscles decrease in absolute weight and also in percentage of protein and in fat during the migration. The most vital constituent is the protein. The muscle proteins are diminished by 30 per cent during the migration without affecting the physiological or histological characteristics of the tissue. So it is obvious that this great excess of protein must exist in the muscle in the form of stored protein. There is a protein storage in the salmon comparable to the similar storage of fats. This stored protein is available during the migration for the synthesis of new protein tissue in the developing ovaries.

THE PREPARATION OF YEAST NUCLEIC ACID.

By EMIL J. BAUMANN.

(From the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven, and the Biochemical Department, University of Toronto.)

A new method for the preparation of yeast nucleic acid is suggested based on the facts: (1) that nucleoprotein is a type of acid protein and is separated into nucleic acid and protein by treat-

Digitized by Google

ment with alkali in the cold; (2) that nucleic acid is soluble in dilute acetic acid; (3) that nucleic acid is precipitated by magnesium sulfate in the presence of hydrochloric acid, as proposed by Slade.

Fresh brewer's yeast is diluted with water and treated with a concentrated solution of sodium hydroxide (100 gm. of alkali per kilo of yeast) and the solution then partially neutralized with hydrochloric acid. The solution is made acid with acetic acid and filtered from the separated protein after the bulk of the precipitate has settled. The filtration is best accomplished with fluted filters or paper pulp. The clear filtrate is treated with 5 per cent of magnesium sulfate and enough hydrochloric acid to cause the nucleic acid to flock out. The precipitated material is the monomagnesium salt and may be dried after washing with alcohol and ether. Yield: 4–7.5 gm. per kilo of yeast.

The analyses for N, Mg, and P are lower than the calculated figures, but the ratios of the three elements correspond accurately with those for monomagnesium nucleate.

The method is applicable to animal nucleic acid with slight modifications.

STUDIES IN ENDOGENOUS URIC ACID METABOLISM.

By HOWARD B. LEWIS, MAX S. DUNN, and E. A. DOISY.

(*From the Laboratory of Physiological Chemistry of the University of Illinois, Urbana.*)

The influence of proteins and various protein derivatives upon the hourly uric acid elimination has been studied in the fasting subject on a purine-free low protein diet. Protein (egg white, cottage cheese) caused a slight rise beginning the 2nd hour after ingestion and increasing to a maximum at the 4th hour. Ingestion of equivalent amounts of nitrogen in the form of amino-acids (glycocoll, alanine) resulted in a sharp rise the 2nd hour after ingestion followed by a prompt return to the normal level. Administration of a second portion of glycocoll following the return to normal caused a like increase in the uric acid excretion. The increased uric acid metabolism was not apparently associated with the phenomena of specific dynamic action of the glycocoll, since aspartic and glutamic acids which probably exert no such

Digitized by Google

action caused increases in the uric acid excretion of even greater magnitude than glycocoll or alanine. Neither urea nor ammonium chloride administered in amounts comparable to the amino-acids had any influence on the uric acid output. These results are believed to indicate that the rise in uric acid excretion following ingestion of protein food is not due mainly to the work of the digestive glands but to stimulation of the cellular metabolism by the amino-acids liberated in the digestion of the protein. Creatinine excretion was not affected in any of the experiments. Uric acid was determined colorimetrically according to Benedict and Hitchcock.

ASPECTS OF HIPPURIC ACID CONJUGATION.

By E. LACKNER, A. LEVINSON, and WITHROW MORSE.

(*From the Nelson Morris Institute, Chicago.*)

The liver is concerned with several types of pairing, yet Salkowski reported that the principal seat of hippuric acid conjugation is resident in the kidney. Salkowski's observations have been repeated by others, and variations in the sweeping statements given by him have had to be made. In the dog, Kingsbury found that there are other organs concerned with this type of pairing. The purpose of the following experiments was first of all to determine whether the liver played a recognizable rôle in hippuric acid synthesis, and, secondly, whether, if this is true, the principle could be utilized in detecting obscure liver involvements in man.

Two male dogs were isolated in metabolism cages and the food, water, and excretions observed over a period of 34 days. The urine was collected in 24 hour quantities. Hepatic involvement was made by the use of hydrazine sulfate, which, according to Underhill and Kleiner and to Gideon Wells, produced liver lesions while leaving the kidneys untouched. The truth of this is vouched for by the chloride figures of the present set of experiments as well as by the evidence of autopsy. Hippuric acid was determined by the Folin-Flanders method; chlorides were determined by the method of Volhard; gasometric amino nitrogen determinations were made upon the hydrolyzed urine to determine the glycocoll participation.

Digitized by Google

The following table gives the résumé of the experiments:

	gm.
(1) Average normal excretion of hippuric acid nitrogen per 24 hours.	
Dog I	0.029
" II	0.032
(2) Average excretion after the administration of 2 gm. of sodium benzoate by mouth.	
Dog I	0.082
" II	0.069
(3) Average excretion after administration of hydrazine sulfate by mouth.	
Dog I	0.043
" II	0.045
(4) Average after injection subcutaneously of 500 mg. of hydrazine sulfate.	
Dog I	0.026
" II	0.030
(5) Average after following this injection with 2 gm. of sodium benzoate by mouth.	
Dog I	0.015
" II	0.014

Averages of the two dogs.

Experiment 1	0.030
" 2	0.075
" 3	0.044
" 4	0.028
" 5	0.014

From these figures, it is evident that normally, an increase in hippuric acid nitrogen after giving sodium benzoate by mouth occurs; that after giving hydrazine by mouth, the hippuric excretion was not affected; that no effect was observed upon the administration of hydrazine subcutaneously, but that *after hydrazine injection followed by sodium benzoate by mouth, less hippuric acid is excreted.*

The kidneys gave every evidence of being normal, as judged by the chloride excretion day by day and also by the condition of the kidney at autopsy. Professor Oscar T. Schultz, director of the Institute, kindly performed the autopsy and reported that Dog II gave wholly normal histological pictures, while Dog I showed some glomeruli and interstitial capillaries engorged, but no other indication of lesion. The livers, on the other hand, showed marked fatty degeneration with central congestion and central necrosis. It is to be concluded, then, that in the dog

Digitized by Google

the excretion of hippuric acid varies with the condition of the liver, the kidney remaining constant.

If it is true that, in the mammal, the liver participates to a major degree in hippuric acid pairing, we should be able to recognize the involvement of the liver by this means. Several normal children were studied, sodium benzoate being given by mouth, and the hippuric acid figures compared with those obtained upon days when no benzoate is given. Invariably there is an increase in hippuric acid excretion. In one case where there was extensive liver involvement (child of 11 years, mitral regurgitation, heart decompensated, liver greatly enlarged), no increase in hippuric acid excretion following administration of sodium benzoate was observed.

CONCLUSIONS.

1. In dogs, there is a marked participation of the liver in the pairing of hippuric acid.

2. This may be determined by administering subcutaneous injections of hydrazine and following these injections by administration of sodium benzoate by mouth, the hydrazine inducing fatty degeneration of the liver, while the kidney remains intact. There is a lessened output of hippuric acid under these conditions.

3. What appears, from a single crucial instance, to be a similar condition in man was observed.

A MODIFICATION OF THE LEWIS-BENEDICT METHOD FOR THE DETERMINATION OF SUGAR IN BLOOD.

By STANLEY R. BENEDICT.

(*From the Department of Chemistry, Cornell Medical College, New York City.*)

A study of the reaction between picric acid and glucose in alkaline solution was undertaken to find conditions under which this reaction will proceed to completion in dilute solution, thus avoiding the necessity of concentrating by boiling, as in the Lewis-Benedict procedure. The results have shown that in no possible concentration of sodium hydroxide is the depth of color directly proportional to the concentration of sugar. This alkali therefore cannot be employed. With increase of concentration of carbonate during the reaction there is increase in depth of color de-

Digitized by Google

veloped almost indefinitely, but high concentrations of carbonate lead to the occurrence of secondary reactions, so that the color developed is again not directly proportional to the concentration of sugar. Thus the concentration of carbonate cannot be increased to cause the reaction to go to completion in dilute solution. It was found, however, that in high concentration of picric acid (greater than a saturated aqueous solution) the reaction with glucose will be definite and quantitative in dilute solution in the presence of a moderate concentration of sodium carbonate. Details of the method thus developed will be published shortly.

A PRELIMINARY REPORT ON THE URINARY EXCRETION OF SUGAR IN THE NORMAL DOG.

By STANLEY R. BENEDICT and EMIL OSTERBERG.

(*From Department of Chemistry, Cornell Medical College, New York City.*)

The excretion of sugar in the urine of a normal dog upon various diets was studied by means of a new method for determination of sugar in normal urine. The technique will be published in detail shortly. The results of the study showed that sugar excretion in the normal dog bears no relation to urinary volume, but is dependent upon food ingestion and the nature of the diet. After food is eaten the curve of sugar excretion promptly rises, reaching a maximum usually in the 4th to 5th hour after the feeding. The curve then falls quite rapidly, reaching a low level during the early morning hours. The sugar excretion is less on a meat diet than on a diet rich in starch, but the curve of sugar excretion follows the same general curve in both cases.

A NEW FORM OF COLORIMETER.

By JOSEPH C. BOCK and STANLEY R. BENEDICT.

(*From Department of Chemistry, Cornell Medical College, New York City.*)

An accurate colorimeter has been developed in which the place of costly and difficultly obtainable prisms is taken by mirrors. The instrument will be described in detail later.

Digitized by Google

ADSORPTION PHENOMENA IN COAGULATION.

By G. H. A. CLOWES.

(*From the Gratwick Laboratory, Buffalo.*)

Physical quality and speed of formation of blood clot appear to depend upon the degree of dispersion of reacting colloids and their initial and final solution concentrations. Much previous work on coagulation is invalidated owing to failure to recognize the extent to which individual coagulation factors are influenced by very slight variations in hydrogen ion content of the medium in the absence of the buffer system.

Precipitated or colloidal barium sulfate, calcium oxalate, etc., may be used to fractionate coagulation factors. Prothrombin is strongly adsorbed by these substances. Adsorption is proportional to the degree of dispersion of the adsorbent and is dependent apparently upon presence of unsaturated lipoids like cephalin, thus indicating probable surface rôle of lipoids in coagulation processes and affording a quantitative method of estimating prothrombin and lipoid.

SALT METABOLISM IN DIABETES.

By A. H. BEARD and L. G. ROWNTREE.

(*From the Department of Medicine, University of Minnesota, Minneapolis.*)

An investigation was undertaken to determine the cause of the gain of weight in diabetes during starvation and on low carbohydrate intake. Gains up to 10 and 12 pounds were encountered during periods of low caloric intake. Edema developed in three cases in a series of twenty-five.

It was found that diabetic patients allowed to use table salt *ad libitum* consumed frequently very large quantities, as high as 40 gm. a day in one instance. Salt and fluid balance charts were constructed and correlated with a weight chart. In some cases it was shown that the gain in weight and development of edema were synchronous with, and in all likelihood dependent upon retention of salt and water.

In one case with restricted sodium chloride intake, but taking large quantities of sodium bicarbonate, retention of fluid and

Digitized by Google

marked gain in weight and edema developed. On withdrawing sodium bicarbonate and allowing sodium chloride loss of weight occurred.

OBSERVATIONS ON THE PERMANGANATE TITRATION OF URIC ACID CONTENT OF BLOOD.

By J. LUCIEN MORRIS.

(From the College of Medicine, University of Illinois, Chicago.)

The determination of the small amounts of uric acid in blood by permanganate titration in acid solution is subject to a relatively large error. This is due to the continued reduction of permanganate after all uric acid has been oxidized. The transitory nature of the pink color end-point gives an error of only 2 to 3 per cent of the 2 mg. of uric acid in the titration following precipitation from urine as zinc urate. The error is 20 to 30 per cent of the 0.2 mg. of uric acid present in the usual quantity of blood taken for analysis. Oxidation of uric acid by permanganate in the slightly alkaline solution of sodium bicarbonate is a much less vigorous reaction and affords an opportunity of measuring the conclusion of the oxidation. The end-point used is the very delicate iodostarch color, which can be applied to the detection of an excess of permanganate because oxidation of potassium iodide does not take place in the weakly alkaline solution until all uric acid has been oxidized.

The precipitated zinc urate and zinc carbonate are dissolved in hydrochloric acid and disodium phosphate is added until all the zinc has been precipitated. To the resulting solution of uric acid in sodium phosphates, with which the zinc phosphate is present as a precipitate, 25 cc. of a saturated solution of sodium bicarbonate, 5 cc. of a 10 per cent potassium iodide solution, and 1 cc. of a 0.5 per cent soluble starch solution are added. 0.002 N permanganate is run in from a burette until the blue color of the iodide of starch appears. Using this procedure, 0.2 mg. of uric acid (the amount found in 20 cc. of blood) can be determined with an accuracy of 5 per cent or better. The results in blood, as well as the application of this new titration to other organic substances, are being studied further.

Digitized by Google

ALVEOLAR CARBON DIOXIDE OF THE NEW-BORN.

By MAX SEHAN.

METHODS OF DETERMINING HYDROGEN ION CONCENTRATION AND BUFFER VALUE (ALKALINE RESERVE).*

By J. F. McCLENDON.

THE ISOLATION AND IDENTIFICATION OF THE THYROID HORMONE.

By E. C. KENDALL.

A SIMPLE METHOD OF MAKING STALAGMOMETERS.

By A. D. HIRSCHFELDER.

THE FOOD VALUE OF SOME OF THE LESS USED PACIFIC COAST FISH.

By F. W. ALBRO and H. A. MATTILL.

*Published in full in *J. Biol. Chem.*, 1918, xxxiii, 19.

Digitized by Google

Digitized by Google

RETURN TO → CHEMISTRY LIBRARY
100 Hildebrand Hall 642-3753

LOAN PERIOD 1 | 2 | 3
[redacted]
4 | 5 | 6

2-HR USE

ALL BOOKS MAY BE RECALLED AFTER 7 DAYS
Renewable by telephone

DUE AS STAMPED BELOW

UNIVERSITY OF CALIFORNIA, BERKELEY
FORM NO. DD5, 3m, 12/80 BERKELEY, CA 94720

Digitized by Google

U.C. BERKELEY LIBRARIES
C036409246

Digitized by Google

Digitized by Google